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Photosynthetic Responses of
Barley (Hordeum vulgare) to Salt Stress

by

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A thesis submitted for the degree of Ph.D.

University of Glasgow
Department of Botany

December 1987

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S U M M A R Y

Agriculture in half of the world's irrigated land is affected by excess salts, mainly sodium salts. Salt-affected soils have a low fertility and tend to yield plants of poor quality.

This project examined the effect on photosynthesis of stressing barley leaves with NaCl. Initially, a comparison was made of the growth (by measurement of the primary leaf) and Na^+ content (by flame photometry) of control and salt-stressed leaves. Those leaves which were stressed by high external NaCl concentrations showed poor growth and contained high concentrations of Na^+ ions. This suggested that barley survives at high salinities by tolerating, rather than simply avoiding, the salt in its external environment.

The effect of NaCl on photosynthetic processes was examined by measuring two main parameters : oxygen evolution and chlorophyll fluorescence. These parameters indicated how effectively both the light and dark reactions of photosynthesis were functioning. In addition, the use of modulated and low temperature chlorophyll fluorescence enabled the distribution of light energy between photosystems I and II to be studied. Using these techniques, NaCl was found to reduce the rate of oxygen evolution and to inhibit the change in distribution of light energy from photosystem II to photosystem I under conditions of over-excitation of photosystem II. NaCl also affected the induction period of photosynthesis, causing a delay in the time taken to reach the S and M chlorophyll fluorescence levels and a decrease in the height of the M peak.

When the water potentials of control and salt-stressed barley leaves were compared using a pressure chamber, NaCl-stressed plants were found to have a lower water potential. To determine whether the osmotic loss of water was responsible for the observed effects of NaCl

on photosynthesis, or whether toxic effects of Na^+ or Cl^- ions were more important, barley leaves were stressed osmotically using solutions of the impermeable substance mannitol. The effects of mannitol stress were then compared to those of NaCl stress; toxic effects of salt ions were found to be the major factor affecting energy redistribution between the two photosystems, but both toxic and osmotic effects were involved in inhibiting growth and oxygen evolution.

In conclusion, high external concentrations of NaCl inhibited photosynthesis in barley. This inhibition was, to a large extent, the result of NaCl affecting energy redistribution between the two photosystems. Although the effect of NaCl was mostly toxic, osmotic effects were also involved.

ABBREVIATIONS

Wherever possible, S.I. units were used in this thesis. Some of the other abbreviations used were:-

ADP	:	adenosine diphosphate
ATP	:	adenosine triphosphate
BSA	:	bovine serum albumin
chl	:	chlorophyll
DCMU	:	3-(3,4 - dichlorophenyl)-1, 1- dimethylurea
DMSO	:	dimethyl sulphoxide
DPGA	:	diphosphoglycerate
G3P	:	glyceraldehyde - 3 - phosphate
MES	:	2 [N-morpholino] ethanesulfonic acid
NADPH	:	reduced nicotinamide adenine dinucleotide phosphate
O.P.	:	osmotic potential
PGA	:	phosphoglycerate
Pi	:	inorganic phosphate
pmf	:	proton - motive force
PSI	:	photosystem I
PSII	:	photosystem II
Q	:	primary electron acceptor of PSII
RPP pathway	:	reductive pentose phosphate pathway
RUBP	:	ribulose - 1, 5 - bisphosphate
RU5P	:	ribulose - 5 - phosphate
S.E.M.	:	standard error of the mean
Tricine	:	N-Tris (hydroxymethyl) methylglycine
μ	:	chemical potential
Π	:	osmotic pressure
ψ	:	water potential

ψ_s	:	solute potential
ψ_p	:	pressure potential
ρ	:	density
$\Delta\psi$:	transmembrane electric field
Δ_{pH}	:	transmembrane proton concentration gradient

INTRODUCTION

1.1 General Introduction

The accumulation of salt in soils is one of the main factors which reduces the agricultural potential of vast areas of land on each continent. These areas tend to yield plants which exhibit, among other symptoms, inhibited growth. An inhibition of plant growth is likely to be due, at least partly, to salt-induced effects on metabolic processes such as protein metabolism, enzyme activity, respiration and photosynthesis.

This project investigates the extent to which salt affects photosynthesis, the process by which solar energy is converted into chemical energy by higher plants, algae and some bacteria. Hopefully, in the future, the results obtained in studies such as this can be combined with the results of studies which investigate the effect of salt on metabolic processes other than photosynthesis. It might then be possible to gain a clear understanding of the mechanisms by which salt affects plant growth.

1.2 Causes and Extent of Salt Accumulation

The natural weathering of rocks releases salts into the surrounding soil. In humid regions, these salts are leached by rainfall to lower layers of soil and, ultimately, by rivers to the sea. However, in arid and semi-arid regions, insufficient rainfall allows only local leaching of salts. Irrigation tends to aggravate the salt problem; evaporation of water from rivers,

canals and reservoirs is high, with the result that the water which is available for irrigation contains a relatively high concentration of salt. The use of fertilisers often increases the level of salt in the soil still further.

For thousands of years, rising levels of salt in soils has caused severe problems for agriculture in arid and semi-arid regions. The slow accumulation of salt in fields resulted in the downfall of the Mesopotamian civilisations; their land, watered by the Rivers Tigris and Euphrates, once fed 25 million people but is now a barren, salt basin (Pearce, 1987). Nowadays, the extent of the salt problem is vast and continues to increase. Large and widespread areas of salt-damaged soils exist on each continent, but are particularly frequent in Australia, Russia, Argentina, China, Iran and India (Szabolcs, 1979). Even highly sophisticated agricultural systems such as those of the western United States have salt problems; one of the areas of the world affected most severely by salt is the Imperial Valley in southern California (Pearce, 1987). It has been estimated that half of the irrigated farms in the world are damaged by salt (Pearce, 1987).

1.3 Sodium Salt Stress

Most of the salt stresses in nature are due to sodium salts. Soils in which the presence of sodium salts (mainly sodium chloride and sodium sulphate) interferes with the growth of the majority of crops are known as saline soils (Szabolcs, 1979). Within the plant kingdom, there is a wide range of survival limits to salt stress. Plants which cannot grow in the presence of high concentrations of sodium salts are called "glycophytes". Plants which can grow in the presence of high concentrations of

sodium salts are called "halophytes". In some plant families, the limit of salt survival tends to be either high or low: low in legumes (peas, beans); medium in cereal grasses (barley, wheat, rye); high in some forage plants (alfalfa), and in plants such as sunflower and sugar beet. The limit of salt survival may be indicated by a cessation of growth or by an actual killing of the tissues in the form of a necrosis or a marginal burn. This is usually followed by a loss of turgor, falling of leaves and finally death of the plant. Bernstein (1964) classifies salinity effects as osmotic, nutritional and toxic. In terms of stress terminology, osmotic and nutritional effects are secondary salt-induced stresses while toxic effects are primary salt injuries.

1.3.1 Secondary Salt-Induced Stresses

1.3.1.1 Osmotic (Water) Stress

There is a direct and inseparable relationship between salt and osmotic (water) stress.

The spontaneous movement of a species j from one location to another always proceeds in the direction of decreasing free energy. The free energy associated with j can be described quantitatively by the thermodynamic parameter known as chemical potential (μ_j), which is defined as :

$$\mu_j = \mu_j^* + RT \ln a_j + \bar{V}_j P + z_j F E + m_j g h \quad \text{equation (i)}$$

where,

μ_j^* is a reference level (constant term)

R is the gas constant

T is temperature

a_j is the activity of j

\bar{V}_j is the partial molal volume of j

P is pressure

z_j is the charge on j

F is the Faraday

E is electrical potential

m_j is the mass of j

g is gravitational acceleration

h is height

For water, which is uncharged ($z_w = 0$), equation (i) reduces to :

$$\mu_w = \mu_w^* + RT \ln a_w + \bar{V}_w P + m_w gh \quad \text{equation (ii)}$$

It can be shown (Nobel, 1974) that :

$$RT \ln a_w = -\bar{V}_w \pi \quad \text{equation (iii)}$$

where π is the osmotic pressure due to the presence of solutes (see below).

From equations (ii) and (iii), the chemical potential of water can be written as :

$$\mu_w = \mu_w^* - \bar{V}_w \pi + \bar{V}_w P + m_w gh \quad \text{equation (iv)}$$

The term "water potential" (ψ) is defined as :

$$\psi = \frac{\mu_w - \mu_w^*}{\bar{V}_w} = P - \pi + \rho_w gh \quad \text{equation (v)}$$

where ρ_w is the density of water $\left(\frac{m_w}{\bar{V}_w} \right)$.

In plant water relations, it has become conventional to express osmotic pressure as solute potential (ψ_s), which is equal in magnitude but opposite in sign to osmotic pressure ($\pi = -\psi_s$). The more concentrated a solution, the more negative is its ψ_s . The pressure term P, which represents the hydrostatic (turgor) pressure of the cell wall on the vacuolar sap, is expressed as a pressure potential (ψ_p). The equation for water potential can thus be re-written :

$$\psi = \psi_p + \psi_s + \rho_w gh \quad \text{equation (vi)}$$

For cells, $\rho_w gh$ can be neglected.

The water potential of pure, free water at standard temperature and pressure is, for convenience, defined as zero. Water tends to move from a region of high water potential to one of lower (more negative) water potential i.e. in the direction of decreasing free energy. The value of the water potential is thus very important since it can be used to predict the direction of water flow between two locations.

Water will flow from the soil into a plant as long as the water potential of the external soil solution is greater than that of the plant tissue. However, since addition of solutes to a solution makes ψ_s more negative and thereby lowers the water potential of the solution, a plant exposed to an external soil solution containing a high concentration of salt may be subjected to osmotic loss of water and, subsequently, to loss of turgor. Although this form of salt stress does not involve actual penetration of cells by the salt, it may still cause severe injuries such as depression of growth and yield (Ehrler, 1960).

1.3.1.2 Deficiency Stress

The decreased growth often observed under conditions of high salinity has also been explained by a suppression of nutrient absorption due to uptake of salt in competition with nutrient ions. Thus, growth of Phaseolus vulgaris and Pisum sativum is decreased by salt stress, but this inhibition can be overcome by supplying potassium (Giorgi, Fichera and Tropea, 1967). Similarly, both growth and calcium uptake are decreased at high salt concentrations in wheat (Poonia, Virmani and Bhumbra, 1972). However, reduced uptake of a nutrient may also occur as a result of a salt-induced decrease in growth (Patel, Wallace and Wallihan, 1975). Consequently, evidence for reduced growth being due entirely to salt-induced nutrient deficiency must be obtained by increasing the nutrient supply and observing whether or not normal growth is resumed. Partial, but seldom complete, removal of growth inhibition has sometimes been reported, but in other cases there is no effect. It seems, then, that nutrient deficiency may sometimes contribute to salt injury but is seldom, if ever, the sole cause of the injury.

1.3.2 Primary Salt Injury

In contrast to secondary salt-induced injuries, primary salt injuries must involve specific toxic effects of the salt either on the external plasmalemma itself or after penetration through the plasmalemma into the protoplasm.

1.3.2.1 Primary Direct Salt Injury

Primary direct salt injury is thought to occur when plant tissue is suddenly exposed to very high salt concentrations, leading to damage to the plasmalemma. This results in changes in the permeability of the plasmalemma which, in turn, leads to the influx and efflux of ions.

1.3.2.2 Primary Indirect Salt Injury

(a) Inhibition of Growth

A significant decrease in growth is often observed in salt-stressed plants which have been shown (by measurement of water potential) to be relatively unaffected by osmotic stress; Gale (1975) puts forward the following explanation.

To survive, a plant must maintain a state of inequilibrium with its environment. If it is growing in normal soil containing the usual low levels of nutrient ions, it must concentrate these ions within its protoplasm until levels suitable for the cell to function normally are achieved. Maintenance of this unbalanced, steady state requires the continuous expenditure of energy. If a plant is growing in saline soil, it must still concentrate nutrient ions but, in addition, it must prevent toxic levels of salt building up in the protoplast. It may do this by excreting the salt using an ion extrusion pump. Excretion of salt in this way requires the expenditure of energy which would otherwise be available for growth; consequently, growth is reduced.

(b) Metabolic Disturbances

The salt-induced inhibition of growth described above is often accompanied by disturbances in metabolic processes such as respiration, protein metabolism and enzyme activity. Although a disturbance in any one of these would be expected to inhibit growth, some of the processes must be inter-related. An inhibition of protein synthesis, for instance, might be expected to decrease the activity of at least some enzymes, and this in turn might affect one or more of the other metabolic processes.

The possible kinds of sodium salt stress injury are summarised in Figure 1. In spite of the distinction which has been made between secondary osmotic effects and primary toxic effects of salt stress, there is usually no categorical evidence pointing to one or the other as the sole cause of the salt injury. Both may contribute to a greater or lesser extent.

1.4 Resistance to Sodium Salt Stress

To resist salt injury, a plant can avoid or tolerate primary and secondary salt stress.

1.4.1 Avoidance of Primary and Secondary Salt Stress

A plant can use any one of three methods to avoid primary salt stress. Firstly, it can passively exclude the salt, provided that the cell is impermeable to the salt. Secondly, it can dilute the salt with water and, thirdly, it can excrete the salt by an active ion extrusion pump. This excretion of salt may also confer resistance to secondary stress due to nutrient deficiency

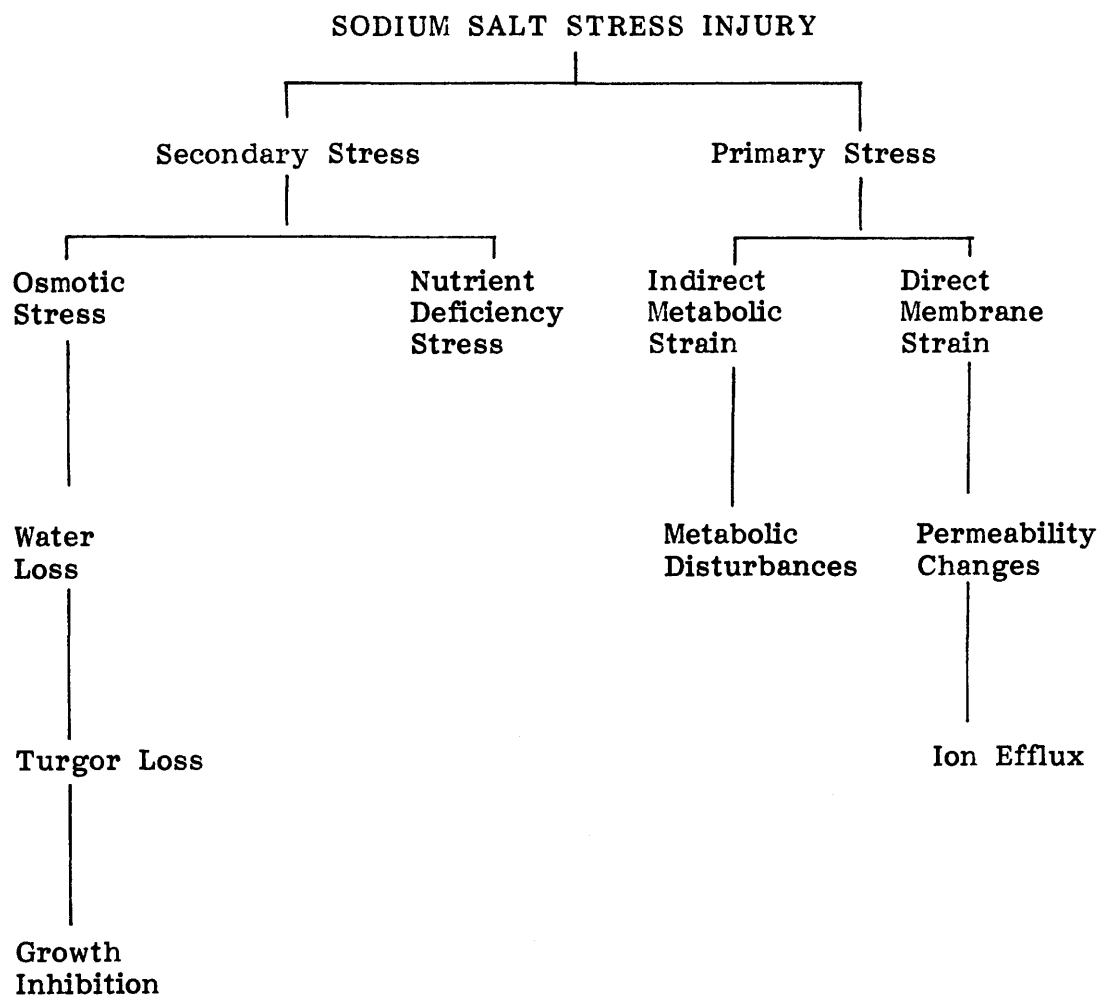


Figure 1. Possible kinds of sodium salt stress injury.

if, for instance, the extrusion pump exchanges extruded Na^+ ions for a deficient ion such as K^+ .

Moderately resistant, non-halophytic plants such as barley owe their salt resistance primarily to avoidance (Greenway, 1965), as do a few halophytes. However, Greenway also notes that when halophytes and non-halophytes are compared, ion accumulation (and therefore tolerance) appears to be a superior mechanism for growth in a saline environment.

1.4.2 Tolerance of Primary Salt Stress

Salt tolerance of primary stress may be accomplished simply by tolerating the ionic stress. For this to occur, however, the protoplasmic organelles have to possess special properties which allow the cell to function normally despite high external salt concentrations.

1.4.3 Tolerance of Secondary Osmotic Stress (Osmoregulation)

Since the plasmalemma is freely permeable to water, cells cannot avoid the osmotic stress of their surroundings. Their first response to salinity is, therefore, a loss of water (and hence turgor) and, if this is severe enough, a loss of ability to grow. Thus, plants must be able to cope with water loss by developing some method which would permit rehydration of the cell, return of cell turgor and recommencement of cell growth. Such a method is possible only as a result of an increase in the content of cell solutes to a level approaching that of the external medium. The plant then avoids water loss by tolerating the same low water potential within its cells as in its environment.

The maintenance of cell turgor by a sufficient increase in cell solutes to compensate for the external osmotic stress is called osmoregulation.

All salt resistant plants must be able to osmoregulate in order to tolerate secondary osmotic stress. In most plants, especially the obligate halophytes, osmoregulation in response to a severe salt stress is achieved mainly, if not solely, by accumulation of inorganic ions from the environment (Wallace and Kleinkopf, 1974). To avoid any adverse effects of high ion concentrations on metabolic processes, these inorganic ions (usually Na^+ , K^+ or Cl^-) are stored inside vacuoles and, in some species, they may later be transferred to salt glands and bladders. Storage of ions in vacuoles, however, causes problems because the resultant lowered water potential of the vacuole will cause the cytoplasm to lose water. This in turn will prevent active metabolism and growth. One way in which the water potentials of the cytoplasm and vacuole may be kept in balance is by the synthesis and accumulation in the cytoplasm of non-injurious or "compatible" organic solutes. A remarkable feature of many angiosperm halophytes is that they accumulate very large amounts of certain metabolites including the amino acid proline (Goas, 1966) and the methylated quaternary ammonium compound, glycine betaine (Storey and Jones, 1975; 1977). Both of these compounds have been suggested to function as compatible cytoplasmic solutes (Stewart and Lee, 1974; Storey and Jones, 1975; 1977). In many halophytic bacteria and algae, accumulation of compatible solutes in the cytoplasm is the main method of osmoregulation. The most extreme example of this kind is provided by the euryhaline green alga Dunaliella. Its osmoregulation on exposure to salt

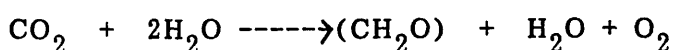
stress is dependent on an accumulation of glycerol (Ben-Amotz and Avron, 1973).

The possible kinds of resistance to sodium salt stress are shown in Figure 2. Although non-halophytes primarily use avoidance to resist salt stress while halophytes primarily use tolerance, both methods almost always contribute in some way to a plant's overall resistance.

1.5 Photosynthesis in Higher Plants

Before studying the effect of salt stress on photosynthesis, it is necessary to understand the underlying photosynthetic mechanisms.

The conversion of solar energy to chemical energy in photosynthesis is of vital importance since it is the ultimate source of food for heterotrophic organisms. Higher plant photosynthesis can be summarised by the empirical equation below.



where CH_2O represents carbohydrate.

The overall process can be divided into two types of reaction : those requiring light (the "light reactions" or "primary processes") and those which can proceed in the light or the dark (the "dark reactions" or "secondary processes"). In the light reactions, light energy is trapped and used to form adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH); in the dark reactions, this

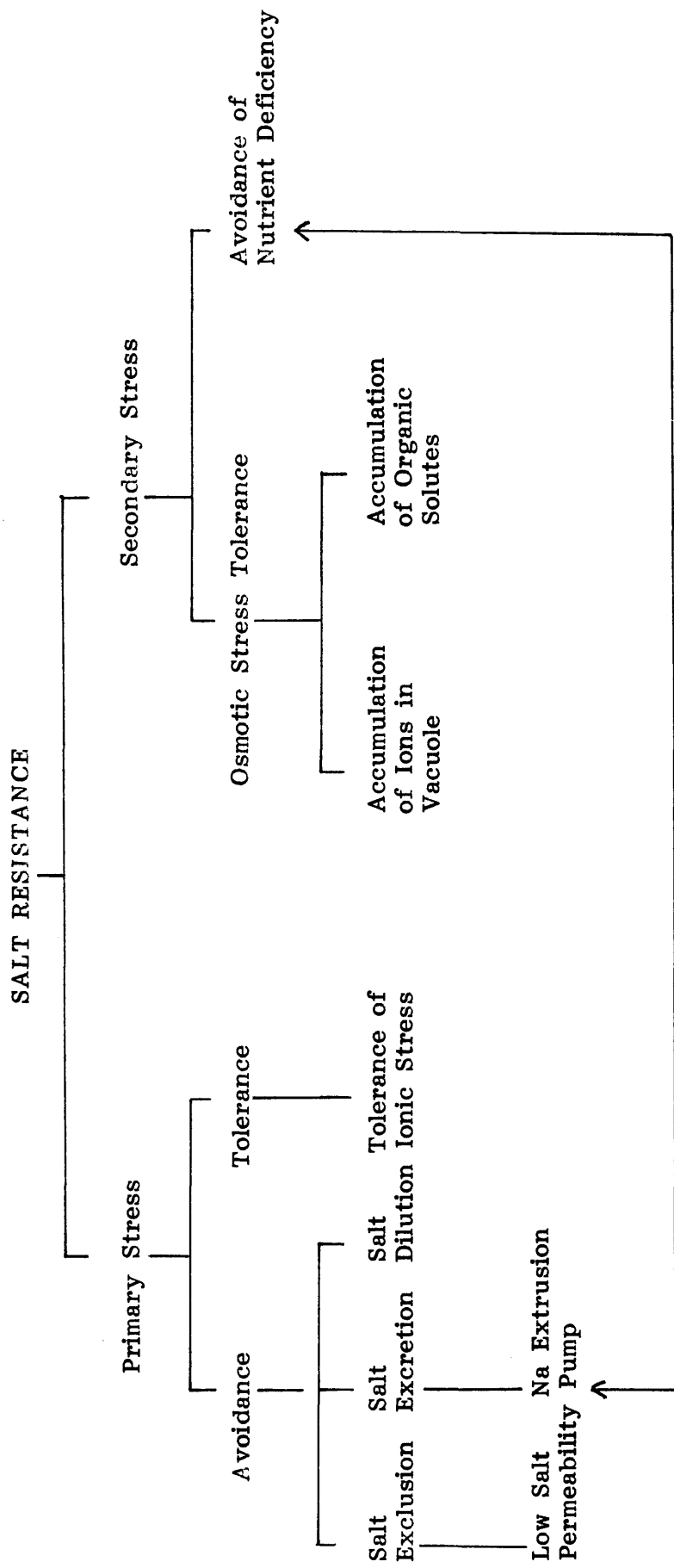


Figure 2. Possible kinds of resistance to sodium salt stress.

ATP and NADPH are used to convert atmospheric CO_2 to carbohydrates.

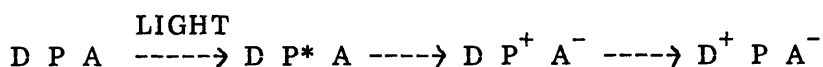
1.5.1 Structure and Function of the Photosynthetic Apparatus

In higher plants, the whole process of photosynthesis occurs in specialised organelles called chloroplasts (Figure 3). Chloroplasts are bounded by an outer envelope consisting of two membranes separated by a space of about 10nm. The envelope contains certain enzymes and is the site of some specific systems for the transport of substances into and out of the chloroplast e.g. in the dark reactions it is the inner membrane of the envelope which acts as a barrier to the free diffusion of metabolites, enzymes and inorganic ions. The envelope encloses the stroma of the chloroplast, in which floats a complex internal membrane structure (the lamellar system). The basic subunits of the lamellar system appear to be double membranes closed within themselves to form flattened vesicles called thylakoids. In places, these thylakoids form stacks called grana, whereas others are longer and may cross the entire stroma. These stromal thylakoids serve to interconnect the grana (Figure 3). The structure of the thylakoid membrane is thought to be based on the fluid mosaic model of Singer and Nicolson (1972) i.e. a lipid bilayer into which various proteins are embedded to form a dynamic, moving structure. The light reactions of photosynthesis occur in or on these thylakoid membranes, while the dark reactions take place in the stroma.

The first step in the light reactions of photosynthesis in higher plants is the absorption of light by chlorophyll a (chl a), chlorophyll b (chl b) and carotenoid molecules located within the

thylakoid membrane. These light-harvesting pigment molecules do not exist freely in the membrane but are bound to very hydrophobic membrane proteins. Chl a, chl b and carotenoids absorb light in the region of the electromagnetic spectrum between 400 - 700nm, each of the three pigments having a different wavelength of maximum light absorption. Chl b and carotenoids pass the light energy which they absorb to chl a. In this way, photosynthesis is able to use a broader area of the spectrum.

Following the results of Emerson and Arnold (1932 a, b) and Emerson (1958), it is now generally agreed that about 300 chl a molecules are concerned with the processing of one quantum of light and that at least 8 quanta are required for the evolution of one molecule of oxygen. The group of 300 chl a molecules is called a "photosynthetic unit". All but one of the chl a molecules in a photosynthetic unit function simply as light harvesters, transferring captured light energy to a single specialised chl a molecule which is located in a specific pigment-protein complex called a reaction centre (Duysens, 1951; Govindjee and Govindjee, 1974). In higher plants, there are two types of reaction centre, called photosystem I (PSI) and photosystem II (PSII); their presence was first inferred by Emerson, Chalmers and Cederstrand (1957). PSI absorbs light energy up to about 720nm, while PSII absorbs only up to about 680nm. Below 680nm, light is absorbed by both systems, PSII absorbing slightly more. Within photosystems I and II, the primary photochemical reaction of charge separation occurs as is shown in the following reaction (Mathis and Paillotin, 1981).



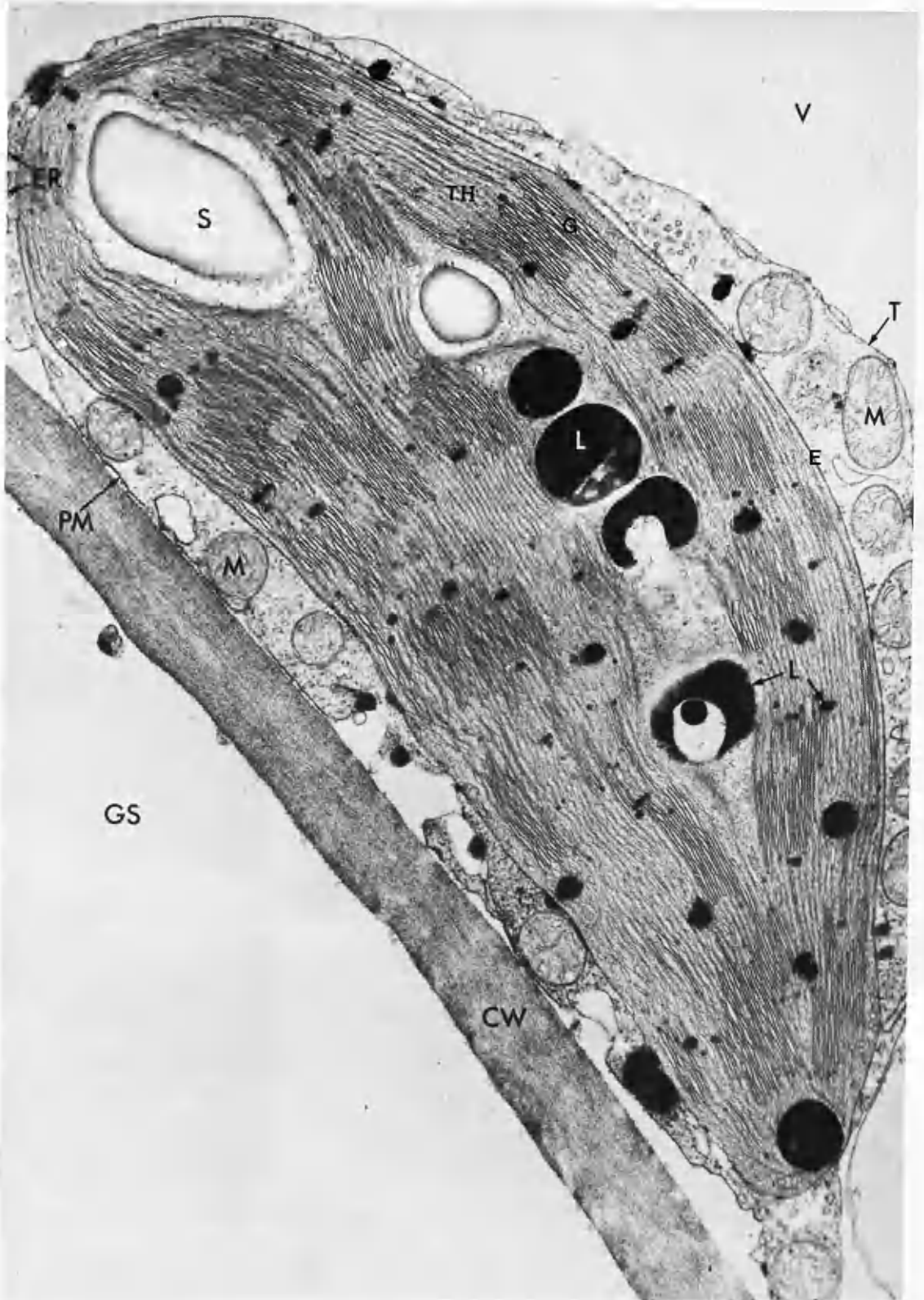


Figure 3. An electron micrograph of a chloroplast isolated from Phaseolus vulgaris. E, envelope; TH, thylakoids; G, granum; S, starch grain; L, lipid droplets; T, tonoplast; V, vacuole; M, mitochondrion; ER, endoplasmic reticulum; PM, plasmalemma; CW, cell wall; GS, gas space. Magnification 154000 Micrograph courtesy of Dr B G Bowes

Light energy is used to promote the reaction centre chlorophyll molecule (P) to an excited state (P^*); this then donates an electron to a neighbouring electron acceptor (A) which is held very close to the reaction centre chlorophyll molecule, and hence P becomes oxidised. Re-reduction of P^+ takes place by oxidation of another electron donor (D). In PSI the reaction centre chlorophyll is known as P700 because a small decrease in absorption occurs at approximately 700nm when P700 is photo-oxidised to $P700^+$. Similarly, the reaction centre chlorophyll of PSII is known as P680.

1.5.2 Non-Cyclic Electron Transport (The "Z-Scheme")

The function of PSI and PSII is to generate the ATP and NADPH required to convert carbon dioxide to carbohydrate in the stroma. Hill and Bendall (1960) suggested that PSI and PSII operate in series and are linked by a c-type cytochrome, cytochrome f, which is reduced when electrons are received from PSII and oxidised when electrons are passed on to PSI. This scheme for photosynthetic electron transport between PSII and PSI is called the "Z-scheme" and, since it was first proposed, a wide range of experimental techniques has been used to investigate in more detail the way in which PSII and PSI are connected. This has led to the discovery of the nature and sequential order of many intermediate electron carriers, although some parts of the Z-scheme are still in doubt. Figure 4 shows the modern complex picture of the Z-scheme; it is drawn on a scale of redox potential, where those compounds with lower mid-point redox potentials (strong reducing agents) are found at the top of the diagram. The "uphill" sections at the reaction

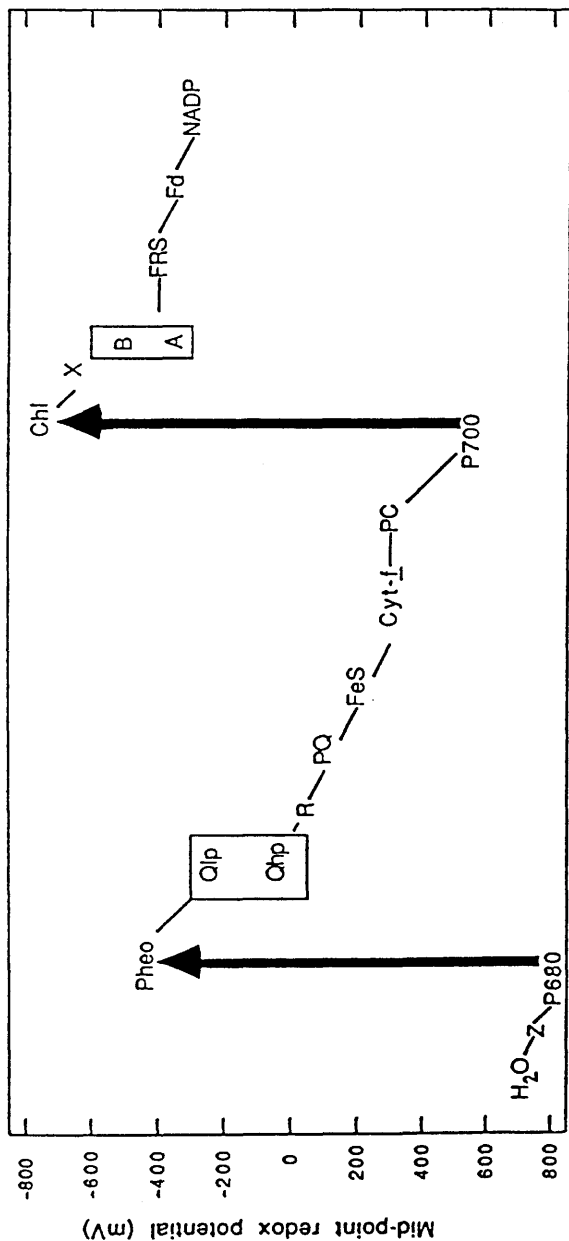


Figure 4. The "Z-scheme" for non-cyclic electron transport.

Z, unknown intermediate; Pheo, pheophytin; Q, quinone; R, intermediate molecule; PQ, plastoquinone; FeS, "Rieske" iron-sulphur centre; Cyt- \bar{f} , cytochrome \bar{f} ; PC, plastocyanin; X, A, B, bound iron-sulphur centres; FRS, ferredoxin-reducing substance; Fd, ferredoxin (Adapted from Hipkins (1984)).

centres require the energy of a photon to reduce an electron acceptor and so make a reducing agent, while the "downhill" sections release energy as electrons pass from stronger reducing agents to relatively weaker ones.

Electrons ejected from P680 pass to the first stable electron acceptor of PSII which is known as Q and which is probably a quinone molecule. Horton and Croze (1979) found evidence to suggest that there are two components of Q with different mid-point redox potentials : the low potential component (Q_{lp}) has a mid-point redox potential of about -250mV while the high potential component (Q_{hp}) has a mid-point redox potential of approximately 0mV. It has been suggested that these two components can act in series or in parallel (Horton and Croze, 1979). There is also some indirect evidence that an electron acceptor is present prior to Q and that it may be a molecule of pheophytin a (Clayton, 1980; Mathis and Paillotin, 1981). However, Horton and Croze (1979) have speculated that Q_{lp} is equivalent to this molecule of pheophytin a.

From Q, electrons are transferred to a pool of plastoquinone (PQ) molecules. PQ accepts electrons from Q via another molecule called R, which is probably a quinone molecule. The role of this intermediate molecule seems to be to allow the accumulation of two electrons (R^{2-}), before both electrons are passed together to a molecule of PQ. At the same time as it accepts two electrons, PQ takes up two protons. Electrons are then transferred to an iron-sulphur (Fe-S) protein known as the Rieske centre. The Rieske centre is oxidised by cytochrome f (Cyt f) which is in turn oxidised by plastocyanin (PC), a copper-containing protein (Kato, 1977). Plastocyanin is believed to be the immediate electron donor to P700 in higher

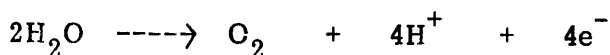
plants.

Absorption of light by P700 causes charge separation as described previously for P680 (Section 1.5.1). Electrons are passed on to an acceptor and are replaced by the feeding in of electrons from plastocyanin. The primary electron acceptor of PSI is not stable, nor is it well characterised but there is some evidence to suggest that it could be a chl a monomer (Baltimore and Malkin, 1980a, b). The secondary electron acceptors X, A and B are all thought to be bound iron-sulphur centres. The way in which electrons pass through these centres is unclear; it is not known if either A or B accepts electrons directly from X or whether both A and B can accept electrons (Okamura, Feher and Nelson, 1982). From the (AB) centre, electrons pass via ferredoxin-reducing substance (FRS) to a soluble iron-sulphur protein, ferredoxin (FD), which is a one-electron carrier. Reduced ferredoxin donates its electron to several different systems, but there are two principal routes. Firstly, the electron may be involved in the reduction of the terminal electron acceptor NADP, via ferredoxin-NADP reductase, to NADPH. On reduction, NADP accepts two electrons and one hydrogen. Hence, the electrons from two reduced ferredoxins must converge to form one NADPH. Alternatively, reduced ferredoxin may donate its electron into the cyclic electron transport system (Section 1.5.3).

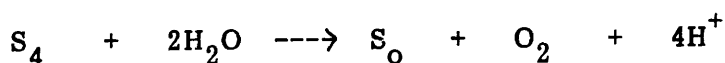
1.5.2.1 Oxygen Evolution

Electrons ejected from P680 are replaced by the oxidation of water molecules. This is the part of the electron transport system which is least well understood. The oxidation of water

requires four electrons to be withdrawn from two molecules of water before one molecule of oxygen is evolved.



Joliot, Barbieri and Chaubaud (1969) and Kok, Forbush and McGloin (1970) investigated the mechanism of oxygen evolution by measuring the yield of oxygen from dark-adapted algae which were suddenly exposed to flashes of bright light. These flashes were short enough to bring about only a single excitation of the PSII reaction centre. A distinctive pattern of oxygen evolution was observed; no oxygen was evolved on the first flash, little on the second but a maximal amount on the third. The sequence continues with a period of four in flash number and gradually damps out until a steady-state level is seen on each flash. A possible explanation of these results was put forward by Kok et al. (1970) and is usually referred to as the "Kok-clock" hypothesis. It suggests that each PSII reaction centre must accumulate four positive charges on its oxidising side before a molecule of oxygen can be evolved. There are thus five possible states in which each PSII reaction centre can exist. These are known as S_0 , S_1 , S_2 , S_3 and S_4 , the last four being formed by the successive loss of single electrons with successive flashes of light. Once S_4 is reached, four electrons are withdrawn from two molecules of water, one molecule of oxygen is released and S_4 reverts to S_0 .



The pattern of proton release is complex in that four H^+ are not

released in one step (i.e. S_4 to S_0) along with oxygen evolution (Fowler, 1977). Instead, there is evidence for release of some of the protons in the other transitions of the S states (Crofts and Wood, 1978).

The observation that the maximum yield of oxygen evolution occurs after only three flashes suggests that, after dark adaptation, most of the PSII reaction centres are in state S_1 , rather than S_0 . It seems that both S_0 and S_1 are stable in the dark, and that during dark adaptation, S_2 and S_3 revert mainly to S_1 and only partially to S_0 .

However, the "Kok-clock" hypothesis would predict that upon illumination with continued repetitive flashes, the sequence would continue indefinitely. Kok et al. (1970) suggested that the dampening out of the sequence might be due to two errors in the change from one state to the next : a state might fail to advance in response to a flash and remain in the same state, or it might suffer a double hit and be moved on two states.

The chemical identity of these charged S states is unknown. Although Cl^- (Izawa, Heath and Hind, 1969) and bicarbonate (Govindjee and van Rensen, 1978) are essential for oxygen evolution to occur, the substance most likely to be involved in charge storage appears to be manganese; it can exist in several different oxidation states and has been implicated in oxygen evolution (Radmer and Cheniae, 1977). The number of electron transfer steps between water and the intermediate species known as Z (Figure 4) is also unclear but there is some evidence that Z may be an immobilised plastoquinol cation radical (O'Malley and Babcock, 1983).

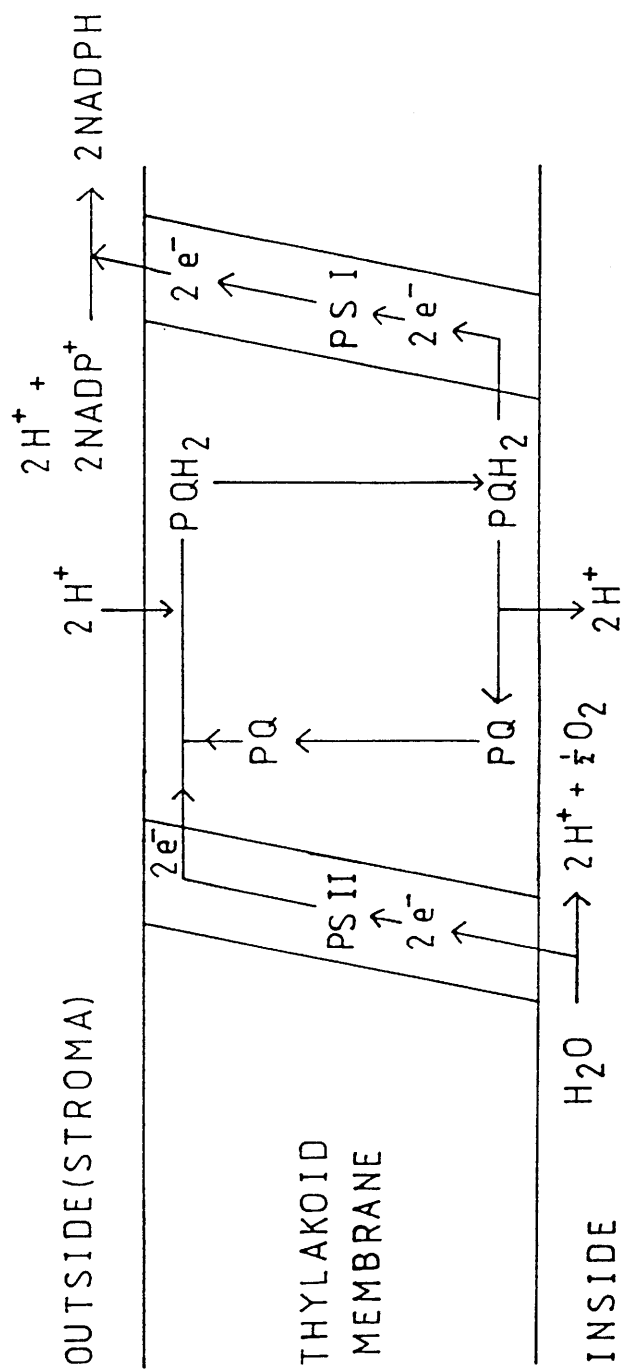


Figure 5. The arrangement of electron transport components in the thylakoid membrane that leads to the movement of protons from the stroma to the thylakoid space along with the transport of electrons from water to NADP. The diagram does not include the possibility that a Q-cycle exists. PQ, plastoquinone.

to the intrathylakoid space lead to the build-up of a transmembrane proton concentration gradient (ΔpH) and a transmembrane electric field ($\Delta \psi$), the combination of which is known as the proton-motive force (pmf). This store of energy tends to expel protons from the intrathylakoid space. The protons move out through a membrane-bound vectorial ATP-synthetase enzyme which takes the energy of the pmf and uses it to form ATP from ADP and Pi. The detailed functioning of the ATP-synthetase is not well understood (Clayton, 1980).

Two observations must be taken into account for the chemiosmotic hypothesis to work : firstly, only closed vesicles can phosphorylate (thylakoid membranes in chloroplasts or the inner membranes of mitochondria in oxidative phosphorylation) and, secondly, the membranes of these vesicles must be essentially impermeable to protons so that they must move out through the ATP-synthetase enzyme.

Jagendorf and Uribe (1966), and Gräber, Schlodder and Witt (1977) have shown that ΔpH alone or $\Delta \psi$ alone can drive ATP synthesis. ΔpH and $\Delta \psi$ are formed at different times after the illumination of chloroplasts; over the first few seconds, ATP synthesis is driven purely by $\Delta \psi$ but, subsequently, only ΔpH is detectable (Ort and Melandri, 1982), with the gradient attaining values of three pH units.

The ratio of protons translocated across the thylakoid membrane to electrons passed down the electron transport chain (the H^+/e ratio) is usually considered to be one, but values of two have also been reported. This inconsistency in the value of the H^+/e ratio may be accounted for by the presence of proton-motive quinone cycles or "Q- cycles" (Velthuys, 1980; Cox and

Olsen, 1982), such as those which are thought to exist in the electron transport chains of mitochondria (Slater, 1983). There is also doubt as to the number of protons which are required to pass through the ATP-synthetase enzyme to form one molecule of ATP. Values obtained range from two to four but there are problems associated with the methods used to make the measurements (Ort and Melandri, 1982).

1.5.3 Cyclic Electron Transport and Photophosphorylation

Cyclic electron transport is driven by PSI only and produces ATP without reducing NADP or oxidising water. Phosphorylation driven by cyclic electron transport was the first type of phosphorylation to be discovered (Arnon, Allen and Whatley, 1954), but its physiological significance is still not clear. This is because, under physiological conditions, it is impossible to distinguish between the ATP produced by cyclic electron transport, and the ATP produced by non-cyclic electron transport. The path of electron flow in the cyclic system is also still unclear but one possible scheme is shown in Figure 6.

1.5.4 Recent Experimental Discoveries

Some features of the light reactions described so far may have to be modified in the near future as a result of recent experimental discoveries. The Z-scheme (Figure 4) implies that one PSII and one PSI are rigidly linked by the electron transport chain. This is an over-simplistic picture for two reasons. Firstly, the ratio of PSII to PSI may not be one (Melis and Brown, 1980). Secondly, the two photosystems are now

thought to be spatially separated along the lateral plane of the membrane; PSII is largely confined to the appressed (granal) membranes, while PSI is restricted to the non-appressed (stromal) membranes (Anderson, 1984; Barber, 1984). The electron carrier plastoquinone (Section 1.5.2) is believed to diffuse laterally from granal to stromal regions, transferring electrons between the two photosystems. However, the diffusion rates for plastoquinone may not be fast enough to allow plastoquinone to travel between the two photosystems in the time required for electron transport (Rich, 1984).

In addition, the photosynthetic unit (Section 1.5.1) may be only a statistical entity rather than a structural one. There is evidence that the light harvesting pigment-protein complex associated with PSII (LHC-II) can move between the two photosystems, and that it is involved in the regulation of energy redistribution by State 1 - State 2 transitions (Section 1.5.5).

1.5.5 State 1 - State 2 Transitions

Efficient photosynthesis requires the simultaneous and equal operation of PSI and PSII. The two photosystems can be unequally excited because their absorption spectra are not identical (Ried, 1972). Unequal excitation of the photosystems is not uncommon under natural conditions because plant growth patterns result in the shading not only of the surrounding species but also of the lower leaves of individual plants (Björkman, 1981). Thus it is advantageous for the redistribution of light energy to occur between the two photosystems so as to maintain a balance in the light energy entering each photosystem. In higher plants, an imbalance in

the light energy entering the photosystems is corrected by increasing the proportion of absorbed light energy that is distributed to the rate-limiting photosystem (Chow, Telfer, Chapman and Barber, 1981). These adaptive changes are referred to as State 1 - State 2 transitions. State 1 is the adaptive state which results from over-excitation of PSI, and is characterised by an increase in the proportion of total absorbed light energy which is transferred to PSII relative to PSI. State 2 results from over-excitation of PSII, and is characterised by an increase in the proportion of total absorbed energy which is transferred to PSI relative to PSII. More details of State 1 - State 2 transitions, and the role of LHC-II in these transitions, will be discussed in Section 3.7.

1.6 The Dark Reactions

The ATP and NADPH produced by the light reactions are used in the dark reactions to fix CO_2 into carbohydrates. In general, this fixation process can occur in two main ways, resulting in either a three carbon (C_3) or a four carbon (C_4) initial product (Hatch, Osmond and Slatyer, 1971).

C_4 plants can be divided into those which produce C_4 acids in the light (e.g. tropical grasses such as maize and sugar cane), and those which show crassulacean acid metabolism (e.g. desert succulents such as pineapple) and accumulate malic acid in the dark (Edwards and Huber, 1981; Osmond and Holtum, 1981).

Most higher plants are C_3 plants and fix carbon dioxide in a complex pathway of enzyme-mediated reactions known as the Reductive Pentose Phosphate (RPP) pathway (Figure 7).

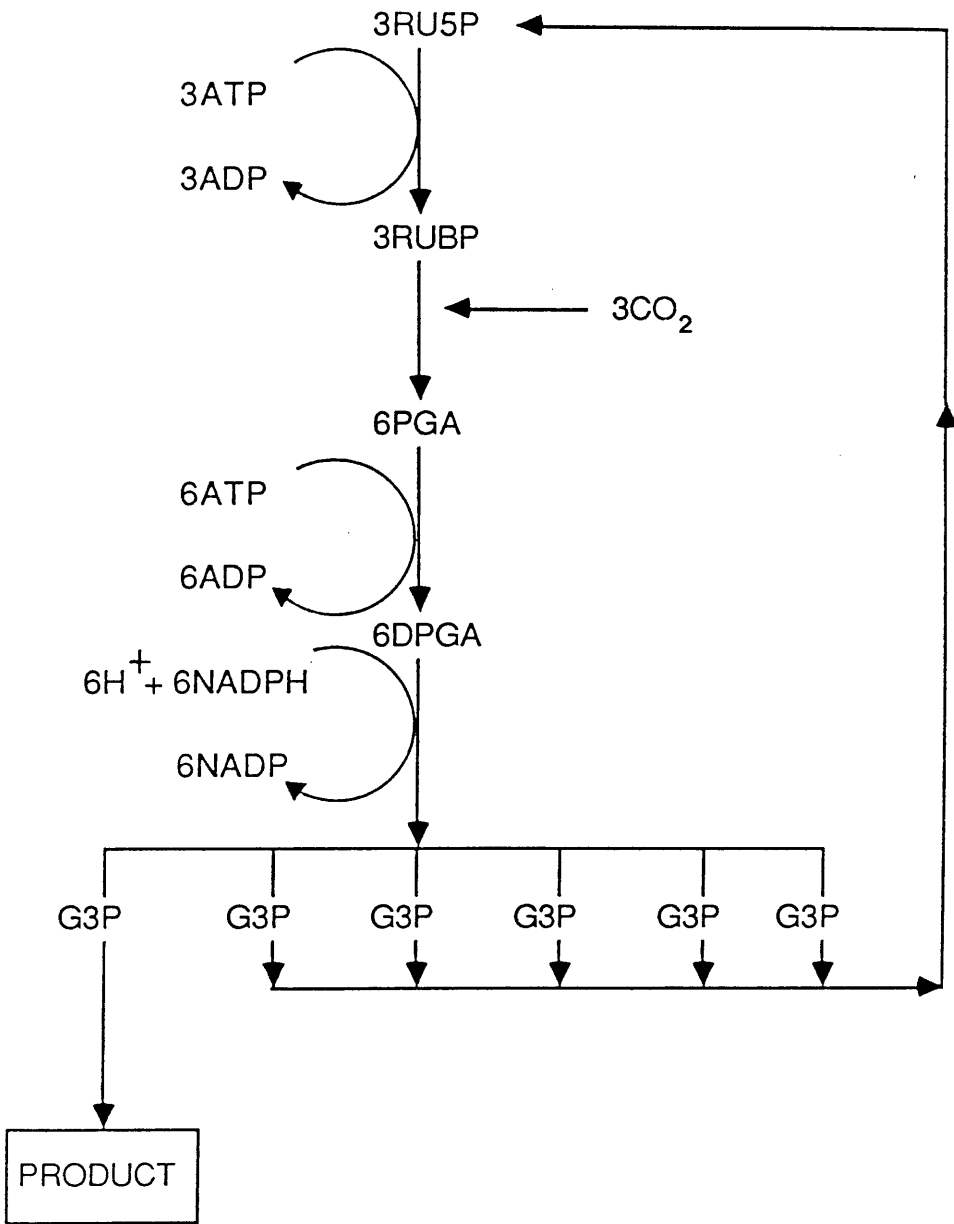


Figure 7. The Reductive Pentose Phosphate (RPP) pathway.
 RU5P, ribulose - 5 - phosphate; RUBP, ribulose - 1, 5 -
 bisphosphate; PGA, phosphoglycerate; DPGA,
 diphosphoglycerate; G3P, glyceraldehyde - 3 - phosphate.

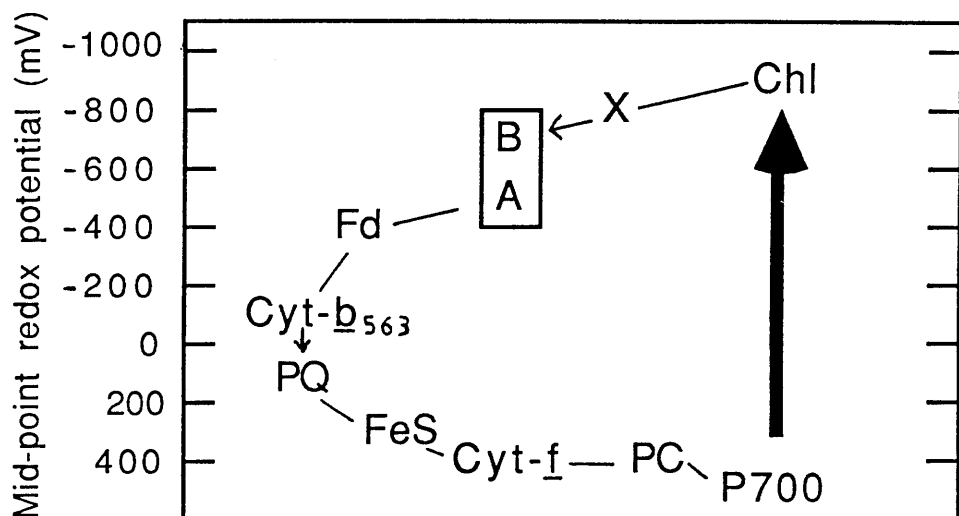


Figure 6. A scheme for cyclic electron flow. Cyt b₅₆₃, cytochrome b₅₆₃. Other symbols as in Figure 4. (Adapted from Hipkins (1984)).

Basically, this pathway involves four reactions. Atmospheric CO_2 first combines with ribulose - 1, 5 - biphosphate (RUBP) to form phosphoglycerate (PGA). After carboxylation, PGA is phosphorylated to diphosphoglycerate (DPGA) at the expense of ATP. The DPGA is then reduced with NADPH to glyceraldehyde - 3 - phosphate (G3P). Some of this G3P constitutes product and may be used for the synthesis of starch or sucrose, but most of it is consumed in the regeneration of RUBP, a reaction which requires ATP (Figure 7).

1.7 Induction

Thus, by providing ATP and NADPH, the light reactions satisfy the needs of the dark reactions. However, there are some circumstances in which the light reactions do not seem to be able to meet all the requirements of the dark reactions immediately. This occurs when plant leaves are strongly illuminated after a period of darkness. Photosynthetic oxygen evolution and carbon dioxide fixation do not immediately reach their maximum steady-state rates. Instead, there is a lag of several minutes. This initial delay is known as the induction period.

Induction periods were first observed by Osterhout and Haas in 1918 and have since been explained in two ways : light activation of enzymes in the RPP pathway, or building-up of substrates in the RPP pathway (Edwards and Walker, 1983). Thus, light may improve catalysis in the RPP pathway either by converting the inactive form of an enzyme to its active form, or by creating a more alkaline environment in the stroma (due to outward movement of protons). Such an environment would allow

those enzymes which were already active in the dark but which have more alkaline pH optima to function more efficiently. Alternatively, induction may be the result of one (or more) intermediates of the RPP pathway having become depleted in the preceding dark period and not being instantly reformed in the light. During the induction period, therefore, there may be an autocatalytic adjustment of its concentration in the light to the level corresponding to the prevailing light intensity. It seems likely that induction is, in fact, due to an interaction between light activation and build-up of substrates; the gradual accumulation of a vital intermediate may also bring about allosteric activation of an enzyme concerned in its formation or consumption.

Edwards and Walker (1983) have proposed specific substrates of the RPP pathway which, in their view, become depleted in the dark and lead to a lag in maximal CO₂ fixation and oxygen evolution. Thus, the lag in oxygen evolution is thought to be due to a depletion in PGA. Oxygen evolution involves the transfer of electrons from water to NADP (Sections 1.5.2 and 1.5.2.1) and, as is shown in Figure 7, this NADP is regenerated by the reduction of PGA, via DPGA, to G3P. This reduction reaction is favoured by a high concentration of PGA and by a high ATP/ADP ratio. However, the ATP produced by the light reactions is also consumed in the formation of RUBP from ribulose - 5 - phosphate (RU5P) and, under conditions of low PGA and high RU5P, the formation of RUBP acts as a powerful sink for ATP. This in turn gives rise to an unfavourable ATP/ADP ratio for PGA reduction so that oxygen evolution is inhibited. Conditions of low PGA and high RU5P would be likely to occur during periods of darkness when the chloroplast

phosphate translocator, which is located on the inner membrane of the envelope, exchanges external Pi for PGA^{2-} (formed by $\text{PGA}^{3-} + \text{H}^+ \rightarrow \text{PGA}^{2-}$) (Figure 8). Upon illumination, however, loss of PGA to the cytoplasm is reduced by the high pH of the stroma which maintains most of the PGA as PGA^{3-} , whereas only PGA^{2-} is transported. PGA gradually builds up and once it reaches a level high enough to overcome the unfavourable ATP/ADP ratio preventing PGA reduction, oxygen evolution can commence.

Similarly, the lag in CO_2 fixation is thought to be due to a depletion in the CO_2 acceptor, RUBP. During the dark, carboxylation can continue for some time leading to a shortage of RUBP but this RUBP cannot be replaced during the dark because its reformation from RU5P requires ATP, and both electron transport and photophosphorylation cease almost immediately in the dark.

1.7.1 The Role of Stomata in Induction

Evolution of oxygen to the atmosphere and uptake of CO_2 from the atmosphere occur through special pores in the leaf epidermis called stomata. Each stoma is surrounded by two specialised, crescent-shaped epidermal cells known as guard cells. Changes in the dimensions and shape of these guard cells, due to changes in turgidity, govern the opening and closing of the stoma, thereby regulating the rate of gaseous exchange.

When higher plants are taken from the dark, their stomata will normally be closed and will begin to open upon illumination (Meidner and Mansfield, 1968). The time course for the opening

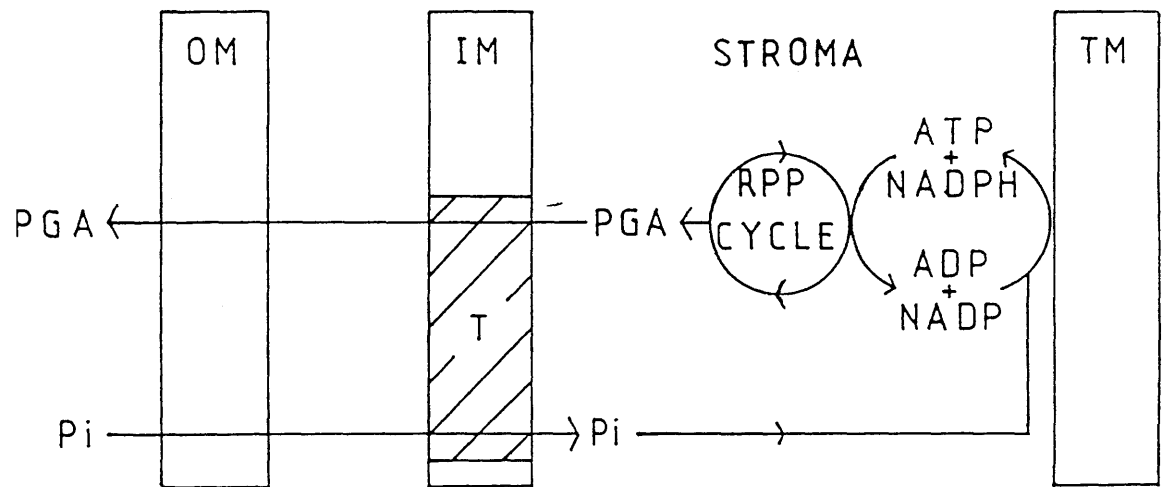


Figure 8. The relationship between Pi and PGA movement. Pi, inorganic phosphate; PGA, phosphoglycerate; OM, outer membrane; IM, inner membrane; TM, thylakoid membrane; T, phosphate translocator.

of stomata is often very similar to the time course for induction, so that induction could simply be the result of an inhibition in the rate of CO_2 diffusion from the external atmosphere into the leaf. However, induction has been observed in aquatic plants which do not possess stomata (Hill and Whittingham, 1953), in leaf discs from which the epidermis has been stripped (Walker, 1976) and in chloroplasts isolated from the leaves of higher plants (Bamberger and Gibbs, 1963). It can therefore be concluded that induction is an intrinsic and fundamental feature of photosynthesis.

1.8 Chlorophyll Fluorescence

Each time a quantum of light energy is absorbed by a light-harvesting chlorophyll molecule, an electron is promoted to a higher energy level i.e. an excited state (Govindjee and Govindjee, 1974). The excess energy of an excited chlorophyll molecule of PSII may be taken up by any one of four competing processes in vivo (Figure 9). These are as follows:

- (1) internal conversion to heat (radiationless decay) - rate constant K_H
- (2) transfer to PSI - rate constant K_T
- (3) trapping by a reaction centre i.e. photochemistry - rate constant K_P
- (4) emission as a light quantum between 685 and 695nm (chlorophyll fluorescence) - rate constant K_F .

Each process is given a rate constant, and the quantum yield of chlorophyll fluorescence (ϕ_F) can be expressed as:-

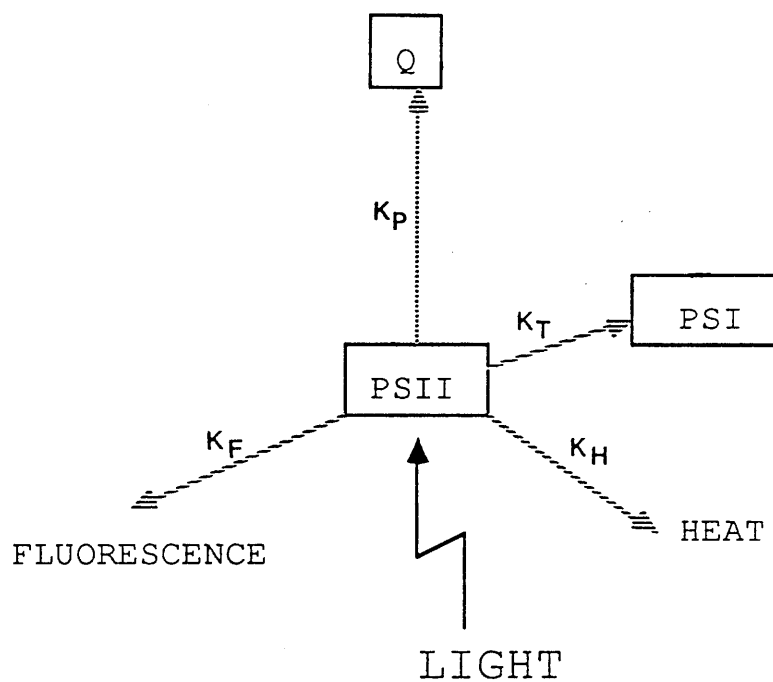


Figure 9. Possible fates for the excitation energy of a chlorophyll molecule of PSII. See text for explanation of K_F , K_H , K_T and K_P .

$$\phi_F = \frac{K_F}{K_H + K_T + K_P + K_F}$$

By definition, the sum of the quantum yields $\phi_H + \phi_T + \phi_P + \phi_F = 1$.

At room temperature, the yield of chlorophyll fluorescence from PSI is low compared to the yield from PSII (Boardman, Thorne and Anderson, 1966; Vredenberg and Slooten, 1967). PSI chlorophyll fluorescence can be detected more easily at cryogenic temperatures (Section 3.8), but the following discussion concentrates on room temperature chlorophyll fluorescence from PSII.

In 1963, Duysens and Sweers suggested that the redox state of Q, the primary electron acceptor of PSII, controlled the yield of chlorophyll fluorescence. When Q is oxidised, a quantum of light trapped by the PSII reaction centre can be used for photochemistry; hence, chlorophyll fluorescence is quenched. However, when Q is reduced, a light quantum trapped by the PSII reaction centre cannot be used for photochemistry, and it is returned to the light-harvesting chlorophylls where it may be emitted as chlorophyll fluorescence. Thus:-

$$\phi_F = \frac{K_F}{K_H + K_T + K_F + K_P A}$$

where A is the number of PSII reaction centres which are associated with oxidised Q. If A increases as a result of more Q becoming oxidised, the yield of chlorophyll fluorescence will be low. However, if A decreases as a result of more Q becoming

reduced, chlorophyll fluorescence will be high. Therefore, the yield of chlorophyll fluorescence is important because it is an inverse measure of the yield of photochemistry.

The measurement of chlorophyll fluorescence from photosynthetic samples is relatively easy. Chlorophyll fluorescence is usually generated by exciting the sample with a white light source. This light is normally transmitted through glass filters which remove wavelengths which are similar to those of the chlorophyll fluorescence being measured. The chlorophyll fluorescence emitted from the sample can then be passed through an interference filter (which removes scattered excitation light), and detected by a photomultiplier or a photodiode. The electrical signal from these detectors can then be passed directly to a chart recorder. This signal is actually a measure of the intensity of chlorophyll fluorescence (F), and involves both the yield of chlorophyll fluorescence (ϕ_F) and the intensity of the exciting light (I) as is shown below.

$$F = \phi_F I$$

1.8.1 Chlorophyll Fluorescence During Induction

The quenching of chlorophyll fluorescence by oxidised Q (q_Q) is not the only quenching mechanism. There are four others: (i) build-up of a transmembrane proton concentration gradient (q_e) (Krause, 1973); (ii) energy transfer to PSI (q_t) (Murata, 1969); (iii) photoinhibition or photodestruction of PSII (q_i) and (iv) non-photochemical quenching by oxidised plastoquinone (q_p) (Quick and Horton, 1984a).

The pattern of chlorophyll fluorescence quenching during induction is known as the Kautsky phenomenon (Kautsky and

Hirsch, 1931), and is both complicated and variable. Work on chloroplasts (Krause, Vernotte and Briantais, 1982; Horton 1983) and leaves (Bradbury and Baker, 1981) suggests that q_Q and q_e are the major quenching mechanisms during induction and these are especially important because they relate to the redox and energy states of the chloroplast respectively. Figure 10 shows a typical Kautsky fluorescence induction curve during constant illumination after a dark period.

1.8.1.1 The Fast Phase

The fast phase (O-P-S) is generally over within 90 seconds. Upon illumination, dark-adapted leaves and isolated intact chloroplasts show a rapid rise to a level F_0 , which is reached before photochemistry is detected. F_0 represents the chlorophyll fluorescence level at a physiological state in which all the intersystem intermediates are oxidised, and it shows that even when the reaction centres are fully active some energy is lost as chlorophyll fluorescence. With the onset of photochemistry, chlorophyll fluorescence rises as Q becomes reduced, until a peak P is reached. P indicates the time at which the ratio of Q reduction to reoxidation is at its maximum. During the chlorophyll fluorescence rise to P there is a dip I-D which is probably an indicator of the early activity of PSI in the oxidation of Q and the PQ pool (Munday and Govindjee, 1969a, b). This dip I-D and the F_0 level are often not seen when chlorophyll fluorescence is measured on a chart recorder because the response time of most chart recorders is too slow to detect them. They are, however, easily seen on an oscilloscope which has a faster response time.

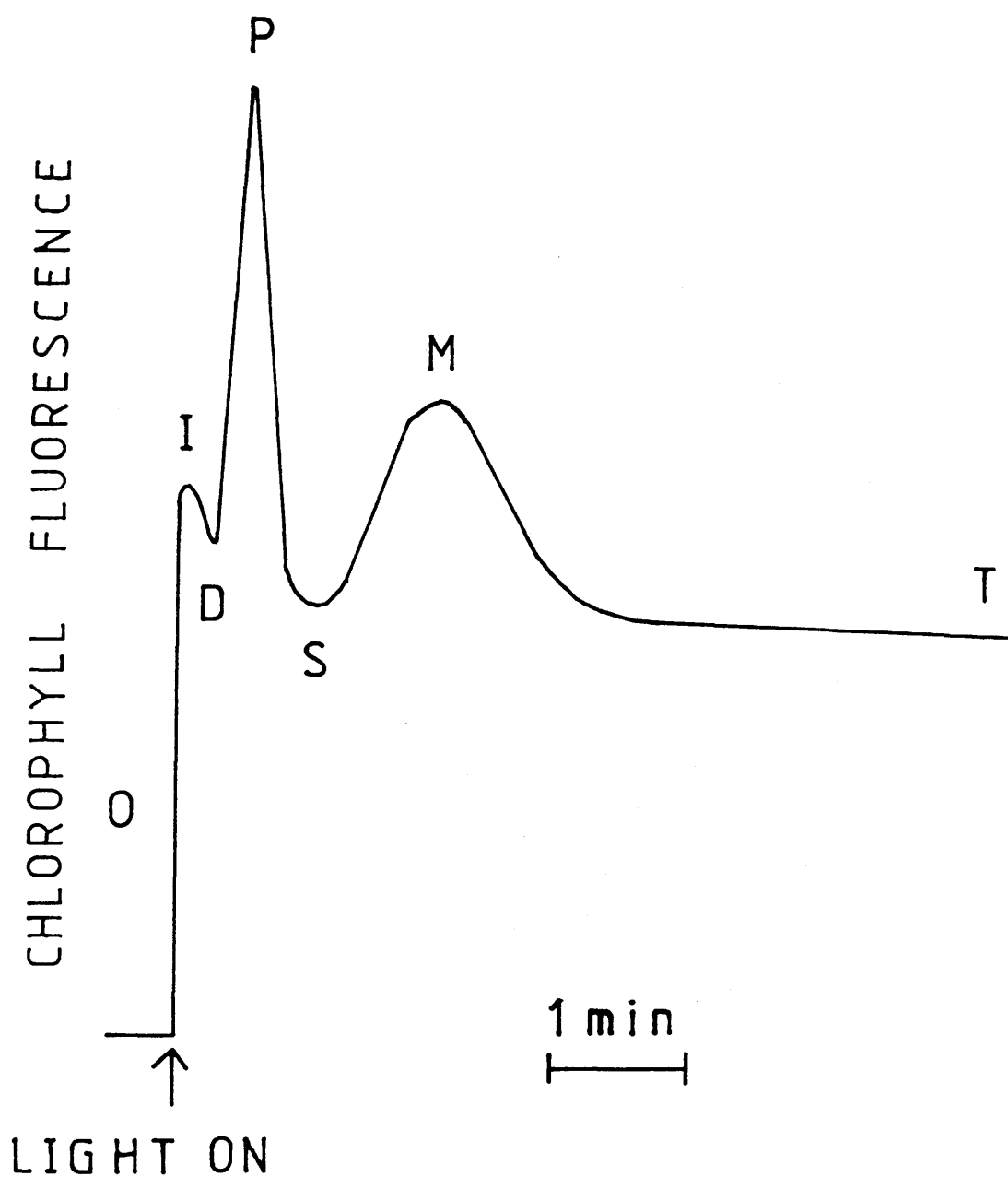


Figure 10. A typical Kautsky chlorophyll fluorescence induction curve during constant illumination after a dark period. See text for explanation of O, I, D, P, S, M and T.

The rapid fall in chlorophyll fluorescence from P to S is thought to be due to both q_Q and q_e ; it seems to result from an increased rate of electron flow from plastoquinone to PSI which increases both the reoxidation of Q and the build-up of a transmembrane proton concentration gradient (Bradbury and Baker, 1981).

1.8.1.2 The Slow Phase

The slow phase (S-M-T) generally lasts several minutes and has been observed mostly in higher plant leaves. The onset of CO_2 fixation is known to be correlated with the onset of the S-M transient (Walker, 1981; Ireland, Long and Baker, 1984). Thus the M peak can be accounted for on the basis of a balance between q_Q and q_e . As CO_2 fixation commences, a sink for ATP is created. If this sink is sufficiently large to produce a large decrease in stromal ATP concentration, the transmembrane proton concentration gradient (and hence q_e) will decrease with a resultant chlorophyll fluorescence increase. However, this rise will be transitory since CO_2 fixation will also increase the rate of non-cyclic electron flow by consumption of NADPH, thus increasing q_Q and causing chlorophyll fluorescence to fall.

After the fall from the M peak, a series of dampening oscillations may occur until a steady-state level of chlorophyll fluorescence is achieved at T. The reason for these dampening oscillations has yet to be explained but a recent hypothesis (Walker, Horton, Sivak and Quick, 1983) has suggested that they result from an interaction between the ATP-producing reactions of the thylakoids and the two kinase enzymes which competitively consume ATP in the RPP pathway (Figure 7). If,

as a result of any disturbance of the steady-state, a pulse of metabolites traversing the cycle gave rise to an increase in RU5P, the consequent ATP consumption by phosphoribulokinase will tend to decrease q_e . In turn, the creation of an unfavourably low ATP/ADP ratio will halt PGA reduction by phosphoglycerate kinase, thereby stopping the regeneration of NADP and causing q_Q to decrease. The decrease in both q_Q and q_e will lead to a rise in chlorophyll fluorescence. Eventually, however, inhibition of PGA reduction will inhibit the regeneration of RU5P, and the consequent reactions will lead to a fall in chlorophyll fluorescence.

1.9 The Aims of the Project

In view of the vast agricultural problems caused by salt, the following research was undertaken in an attempt to answer three questions:

- (i) to what extent does salt affect photosynthesis?
- (ii) which of the underlying mechanisms in the photosynthetic process are affected by salt?
- (iii) is the effect of salt toxic, osmotic or both?

Much of this research involved stressing barley with NaCl. Barley was chosen for two reasons: firstly, it is moderately salt resistant and, secondly, it is a cereal and cereals are economically the most important agricultural plants in the world. NaCl was chosen as the form of salt stress because most of the salt stresses in nature are due to sodium salts, particularly NaCl.

The effect of NaCl on photosynthesis in barley was determined mainly by studying two photosynthetic parameters:

- (a) oxygen evolution - the rate of oxygen evolution depends on the rate of CO_2 fixation which, in turn, depends on the availability of ATP and NADPH. Oxygen evolution is, therefore, a measure of how effectively the light and dark reactions are interacting, and indicates how well the photosynthetic process is working as a whole.
- (b) chlorophyll fluorescence - since the fast phase of chlorophyll fluorescence during induction is thought to be due mainly to electron transport events, and the slow phase is thought to be due mainly to the onset of CO_2 fixation, measurement of chlorophyll fluorescence during induction can indicate how effectively either the light reactions alone or the dark reactions alone are operating. In addition, chlorophyll fluorescence can be used to study individual components of the electron transport chain e.g. the redox state of Q, the primary electron acceptor of PSII.

C H A P T E R 2

MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Barley seeds (Hordeum vulgare L. c.v. Golden Promise) were obtained from Sinclair McGill, Ayr, Scotland and planted in potting compost (Scottish Agricultural Industries, Livingston, West Lothian), perlite, vermiculite (both obtained from Silvaperl Products Ltd., Harrogate, North Yorkshire) or sand. The seeds were watered using Knopp's nutrient solution (see Appendix) and grown in a constant temperature growth room (22°C) under a regime of 12 hours light, 12 hours dark. Illumination was provided by two Thorn 400W MBIF/BU Mercury Vapour lamps, giving an irradiance of 26Wm^{-2} .

Seedlings were harvested 6 to 10 days after planting. In all experiments involving detached leaf pieces, the pieces were always 2cm leaf segments cut immediately below the top 0.5cm of the primary leaf.

2.2 Measurement of Light Intensity

Light intensity was measured using a Model 40X Opto-Meter (United Detector Technology, Santa Monica, California, U.S.A.). The meter was equipped with a radiometric filter which provided an essentially flat spectral response from 450 - 910nm.

2.3 Stress Treatments of Barley Leaves

2.3.1 Salt Stress

Barley seeds were planted in perlite in 38cm x 25cm seed trays and watered using Knopp's nutrient solution. After four days the seedlings were stressed with nutrient solution supplemented with varying concentrations of NaCl. The appropriate salt solution (1.5 litres) was applied to the seedlings and allowed to pour through the drainage holes in the seed trays. This ensured that the root-zone was exposed to a salinity close to that of the watering medium. After this initial salt treatment the leaves were watered lightly each day with 300ml of the appropriate salt solution.

2.3.2 Osmotic Stress

Barley seedlings were exposed to osmotic stress in the same way as they were to salt stress except that the nutrient solution was supplemented with varying concentrations of mannitol.

2.4 Measurement of the Osmotic Potential of Stressing Solutions

The osmotic potentials of salt- and mannitol-stressing solutions were estimated by the cryoscopic method which involves measuring the freezing points of solutions. Full details of the principles on which cryoscopy is based are given in Crafts, Currier and Stocking (1949). The freezing points were measured using an electronic temperature measuring instrument,

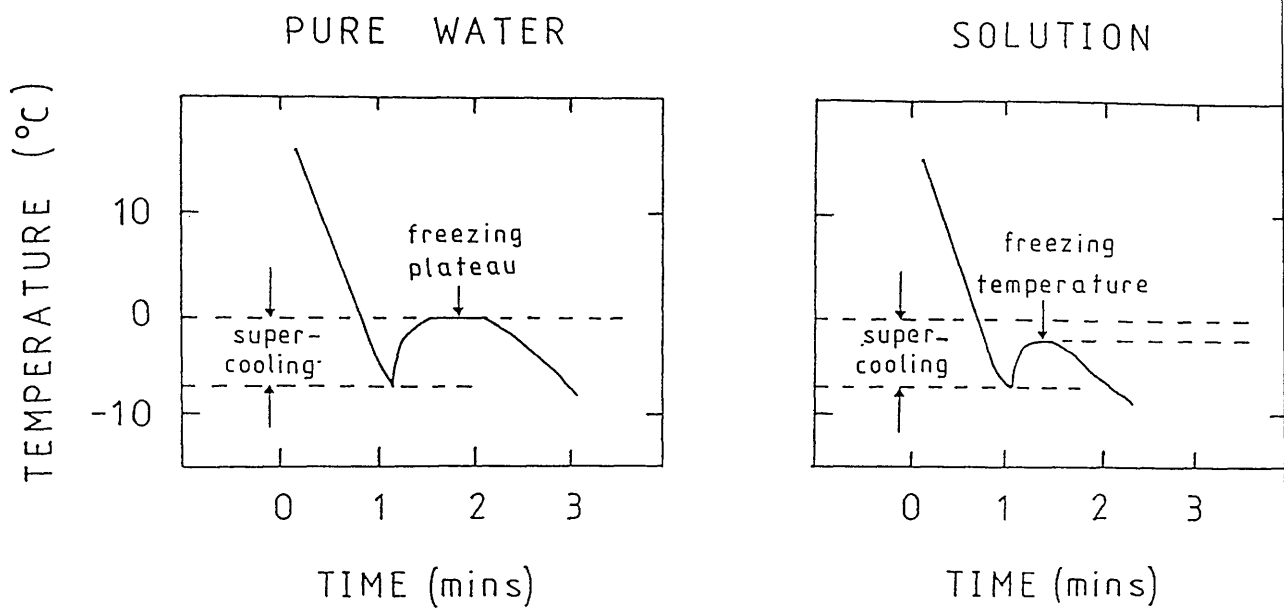


Figure 11. Diagrammatic temperature time curves for pure water and for a solution.

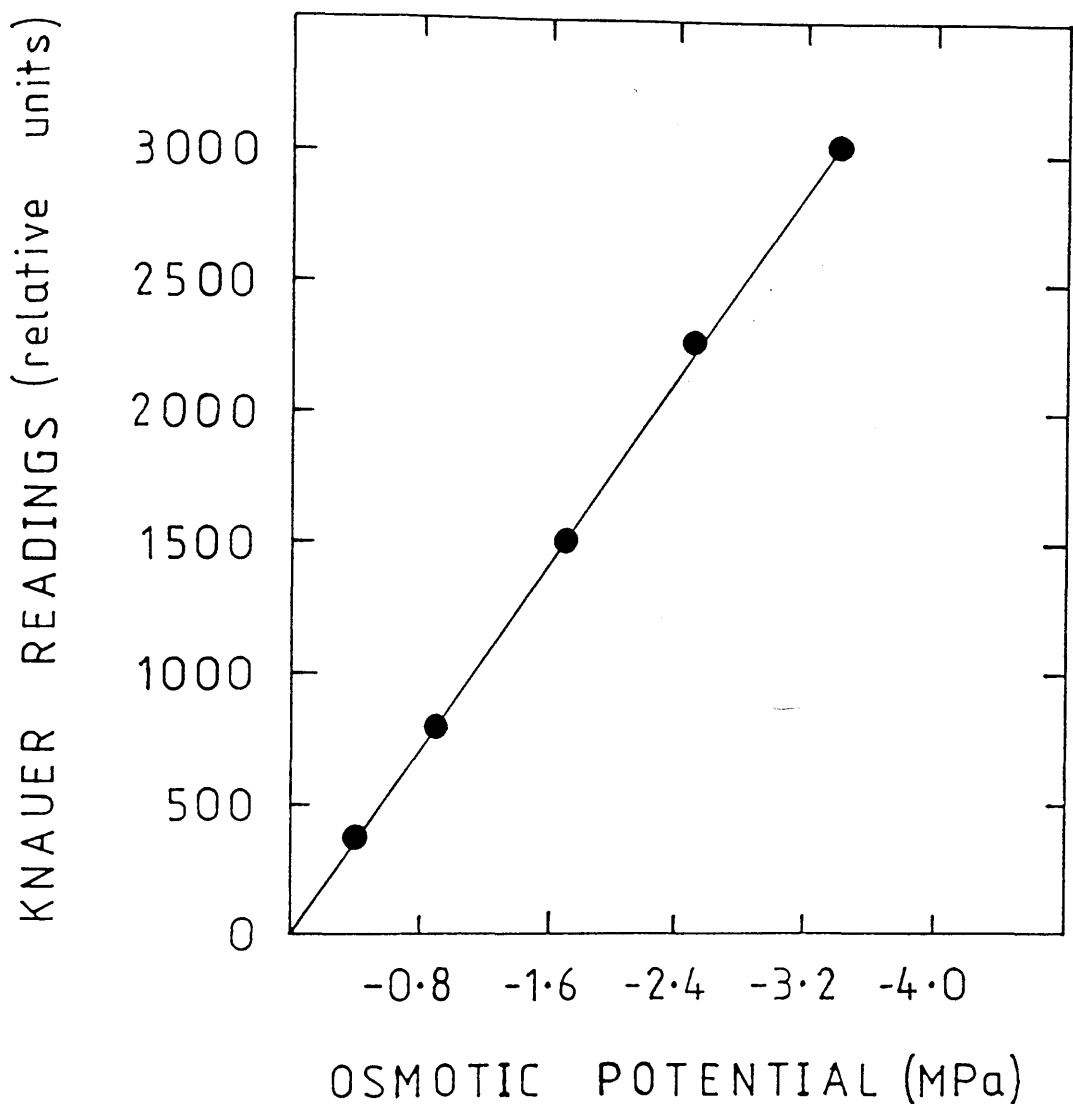


Figure 12. Calibration graph for the determination of the osmotic potentials of sodium chloride solutions by freezing point depression. Each point represents the mean of 5 determinations. Standard error bars were within the symbol size. Osmotic potentials of the calibration solutions were derived from the tables of Wolf et al. (1985).

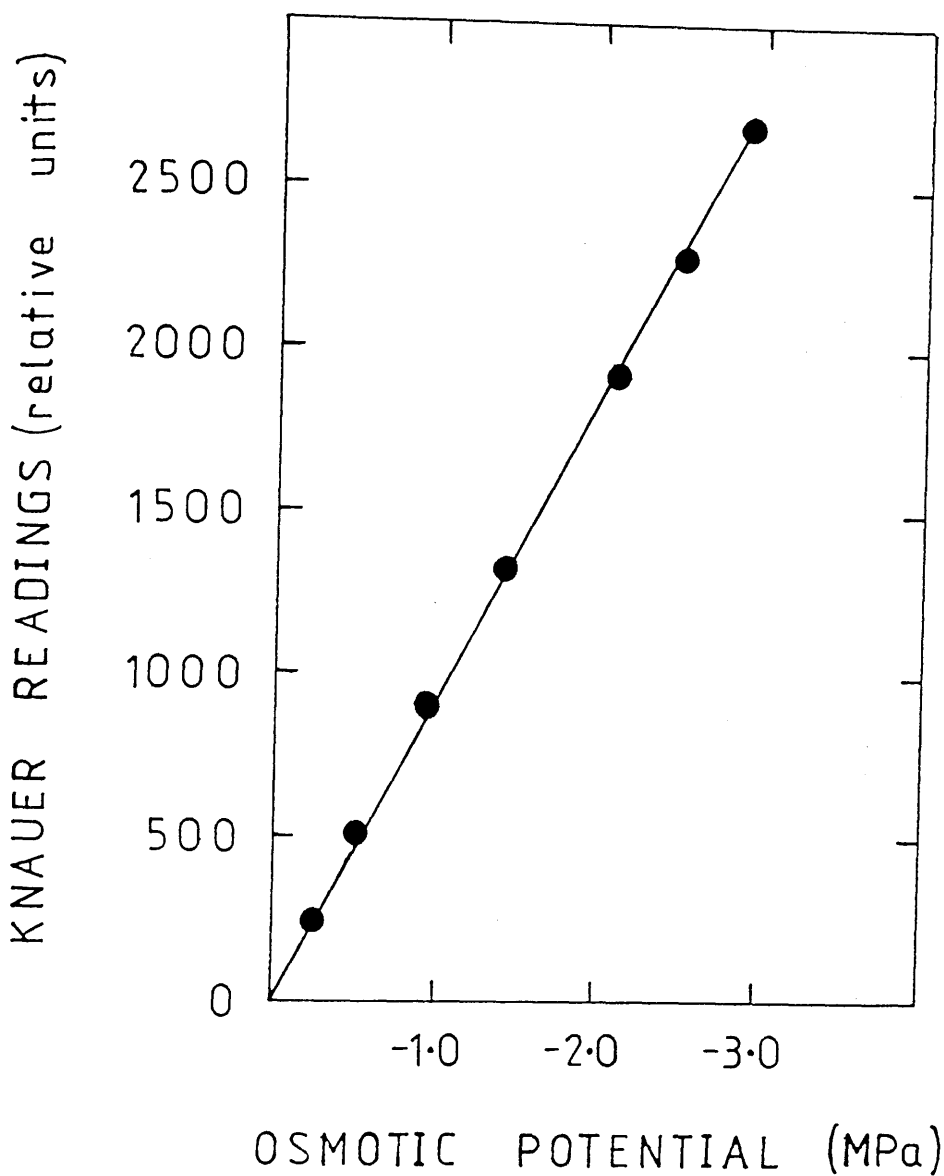


Figure 13. Calibration graph for the determination of the osmotic potentials of mannitol solutions by freezing point depression. Each point represents the mean of 5 determinations. Standard error bars were within the symbol size. Osmotic potentials of the calibration solutions were derived from the tables of Wolf et al. (1985).

2.5 Measurement of the Water Potential of Barley Leaves

The water potentials of salt- and mannitol-stressed barley leaves were measured using a Scholander-type pressure chamber (Scholander, Hammel, Hemmingsen and Bradstreet, 1964; Scholander, Hammel, Bradstreet and Hemmingsen, 1965) produced commercially by Chas. W. Cook and Sons, Ltd., Perry Bar, Birmingham.

2.5.1 Working Principle of the Pressure Chamber

When a leaf or shoot is placed in a pressure chamber with only the cut petiole or stem protruding through an airtight seal to the open air, it is possible to pressurise the leaf until sap just begins to emerge through the cut xylem tissue outside the chamber. The equilibrium pressure at which the cut xylem surface is just wet is called the balance pressure. The balance pressure is numerically equal to the water potential of the leaf but with opposite sign.

2.5.2 Procedure for Determining Leaf Water Potential Using the Pressure Chamber

A diagram of the pressure chamber is shown in Figure 14. Before measuring the water potential of a primary leaf of barley, the coleoptile was removed carefully. The leaf was then sealed immediately into the pressure chamber (internal dimensions 3cm x 30cm) by inserting the leaf base through a slit in a rubber bung and then fitting the rubber bung tightly into the lid of the

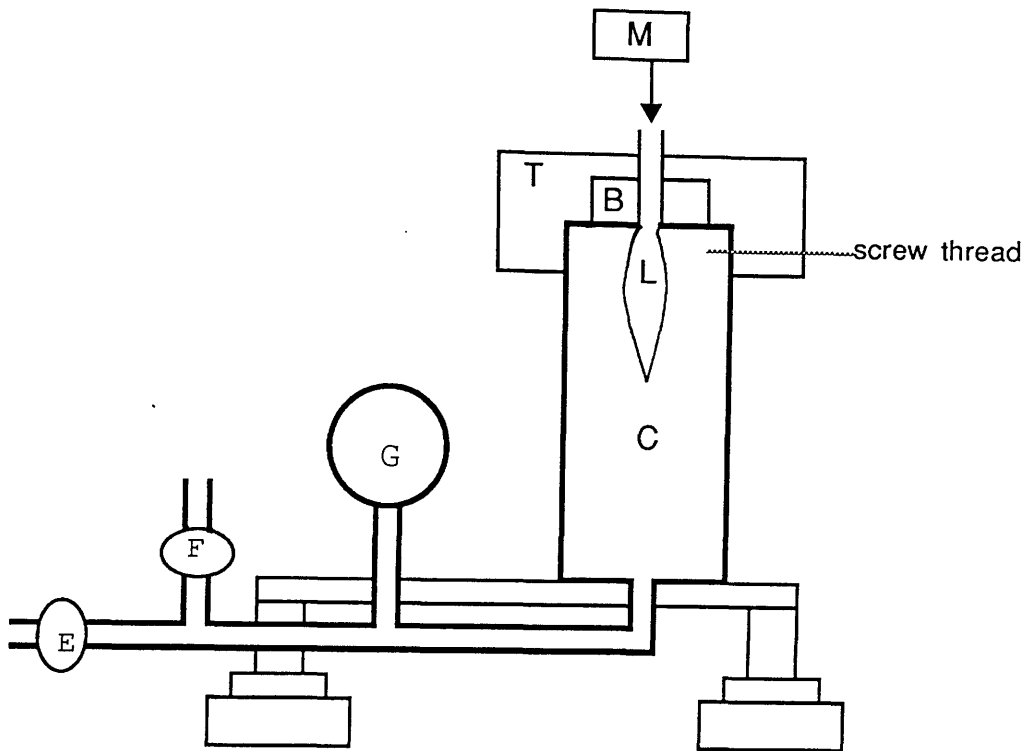


Figure 14. The use of the pressure chamber to measure the water potential of a barley leaf. The leaf (L) was inserted into the pressure chamber (C) through the rubber bung (B), which was placed in the chamber top (T) and sealed with silicone grease. The chamber pressure was increased or decreased in known amounts by use of inlet (E) and exhaust (F) valves and the pressure gauge (G). A dissecting microscope (M) was positioned above the chamber so that sap expression could be seen more easily.

chamber. Silicone grease was used on the bung to ensure an adequate seal between stem and rubber. The length of leaf protruding from the pressure chamber was always 1mm to minimise errors caused by non-pressurisation of part of the sample (Slavik, 1974). A dissecting microscope was positioned above the chamber so that sap expression could be seen more easily.

The pressure chamber was pressurised using oxygen-free nitrogen and the pressure inside the chamber was monitored by a Wika pressure gauge (range 0 to 4.14MPa). Water loss from the sample was minimised by admitting gas into the chamber through a pad of moist paper towel. The pressure inside the chamber was raised in 0.034MPa stages and held for 2 minutes at each stage to allow equilibration to occur. After the first expression of sap the pressure was released by 0.1 to 0.2MPa and then slowly increased again. When the pressure at which sap reappeared on the cut surface was the same on successive measurements the balance pressure was noted.

2.6 Determination of the Sodium Content of Barley Leaves

2.6.1 Extraction of Sodium from Leaf Pieces

Barley leaf pieces (500mg) were surface-washed with distilled water and then dried to constant weight in an oven at 100°C. The dried leaves were ground thoroughly in 2ml of 5% HCl using a mortar and pestle. The resultant leaf extract was boiled on a hot plate until almost dry, made up to 10ml with distilled water

and then centrifuged at 13400 x g for 5 minutes in an MSE Micro Centaur. After centrifugation the supernatant was kept for analysis of sodium content (Section 2.6.2).

2.6.2 Estimation of Sodium

The sodium content of the supernatant obtained in Section 2.6.1 was measured using a flame photometer (Evans Electroselenium Ltd. (E.E.L.), Halstead, Essex). The flame photometer works on the principle that a metallic salt, drawn into a non-luminous flame, ionises and emits light of characteristic frequency.

The supernatant was drawn into the flame of the photometer in the form of a finely atomised spray. The emitted light was passed through an E.E.L. sodium filter (which transmitted wavelengths between 550 - 600nm) onto a photocell, the output of which was used to deflect a galvanometer.

The flame photometer was calibrated using standard solutions of sodium chloride and the calibration curve obtained (Figure 15) was used to calculate the concentration of sodium in the supernatant. Using the values for fresh and dry leaf weight, the concentration of sodium in the leaves was then expressed as mM Na (tissue water).

2.7 Determination of the Mannitol Content of Barley Leaves

The internal mannitol content of control and mannitol-stressed leaf pieces was determined by gas-liquid chromatography (G.L.C.).

GALVANOMETER READINGS

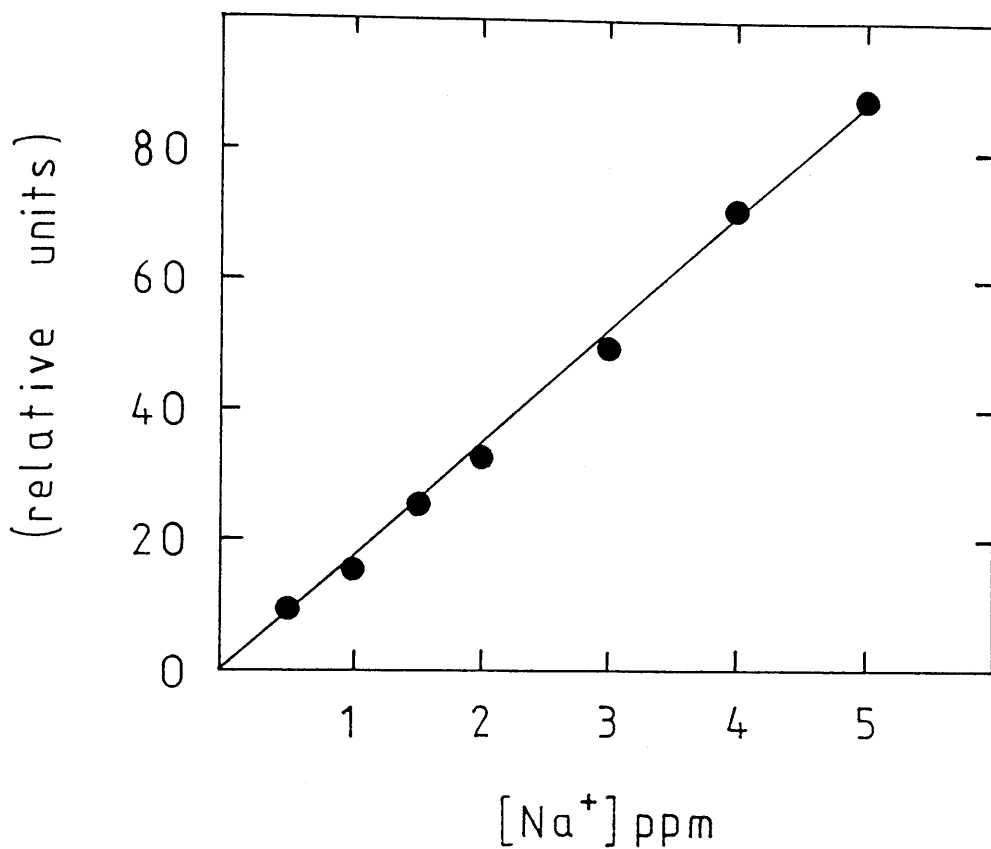


Figure 15. Calibration graph for the determination of $[\text{Na}^+]$ by flame photometry. Each point represents the mean \pm S.E.M. of 3 determinations. Standard error bars were within the symbol size.

2.7.1 General Principles of G.L.C.

In G.L.C., the components of a mixture are separated in a column which contains a stationary liquid phase. The sample is injected onto the top of the column (which is contained in an oven) where it is volatilised and carried through the column by a stream of inert carrier gas. Partition of the components of the sample occurs between the stationary liquid phase and the mobile gas phase, and the time between introduction and elution of a component (the retention time) is related to the affinity of the component for the stationary phase; those components which are most soluble in the liquid phase emerge from the column last and those which are least soluble in the liquid phase emerge first. After elution from the column, the separated components are carried in the stream of gas through a detector. Frequently, the detector is mass-sensitive, so that the magnitude of the signal it produces is proportional to the mass of the component which passes through the detector per unit time. The signals from the detector produce a chromatogram on a chart recorder. The chromatogram consists of a series of distinct peaks, each of which corresponds to a particular component of the mixture. The amount of each component is proportional to the peak area that it produces on the chromatogram. Therefore, by comparing the retention times of known samples with the retention times of the unknown components, the identity of the unknown components may be assumed. In addition, by injecting a known amount of internal standard into the column along with the sample, the amount of each component can be estimated.

2.7.2 Extraction of Mannitol from Leaf Pieces

Barley leaf pieces (0.1g) were surface-washed thoroughly with distilled water and placed in a test tube containing 5ml of distilled water. The leaves were boiled for 15 minutes to denature any enzymes which might degrade mannitol. The contents of the tube were then poured into a mortar and pestle, the leaves were ground thoroughly and the resultant leaf extract was centrifuged at 450 x g for 10 minutes in a Hettich EBA 3S bench-top centrifuge. After centrifugation, the supernatant was poured into a flask containing myo-Inositol (0.05ml of a 20mg. ml⁻¹ solution) as an internal standard.

2.7.3 Acetylation

G.L.C. is only applicable to volatile substances. Sugar alcohols such as mannitol and myo-Inositol have multiple hydroxyl groups in their structure; these hydroxyl groups render mannitol and myo-Inositol non-volatile due to hydrogen bonding. Acetylation of these hydroxyl groups using acetic anhydride eliminates hydrogen bonding and ensures that mannitol and myo-Inositol volatilise readily when injected onto the column. The acetylation procedure outlined below is a modification of the technique of Blakeney, Harris, Henry and Stone (1983).

The supernatant obtained in Section 2.7.2. was rotary evaporated to dryness, resuspended in 1ml of dimethyl sulphoxide (DMSO) and poured into a 14ml septum-cap vial. 1-Methylimidazole (0.2ml), followed by acetic anhydride (2ml), was

added to the vial and the contents were mixed vigorously using a vortex mixer. The 1-Methylimidazole acts as a catalyst in the acetylation procedure. After exactly 10 minutes at room temperature, 5ml of water was added to decompose the excess of acetic anhydride and the contents were again mixed thoroughly. When the contents had cooled to room temperature, 1ml of dichloromethane was added, the contents were mixed thoroughly and the mixture was centrifuged at $4000 \times g$ for 5 minutes in a MSE minor "S" bench-top centrifuge. After centrifugation, the dichloromethane (which contained the acetylated mannitol and myo-Inositol) formed a layer at the bottom of the vial and was removed using a Pasteur pipette.

2.7.4 Separation of the Acetylated Components

Gas-liquid chromatography was carried out using a Packard 430 chromatograph fitted with a glass-packed column (2m x 2mm, i.d.). The column was packed with 3% SP-2330 on 100/120 mesh Supelcoport. The eluted components were detected by a flame ionisation detector which burnt a mixture of H_2 and air. Argon was used as the carrier gas at a flow rate of 20ml. min^{-1} . Routinely, $2\mu\text{l}$ samples were injected. Following injection of the sample, the oven temperature was kept at 200°C for 5 minutes and then raised at $2^\circ\text{C. min}^{-1}$ to 220°C , where it was held for 15 minutes. The injection port and the detector were heated to 270°C .

Before comparing the internal mannitol content of barley leaves, three control experiments were carried out as described below.

- (1) To verify that the flame-ionisation detector of the gas-liquid chromatograph was giving a linear response to increasing concentrations of mannitol, 1 to 6mg of mannitol were dissolved in 5ml of distilled water. The solutions were then added to flasks containing myo-Inositol (as in Section 2.7.2) and the acetylation procedure was carried out as described in Section 2.7.3.

Figure 16 shows the response of the flame-ionisation detector.

- (2) Myo-Inositol has been found in minute amounts as a natural constituent of a very small number of angiosperms (Holligan and Drew, 1971). The presence of endogenous myo-Inositol would obviously affect its use as an internal standard. It is therefore important to determine if and at what concentration myo-Inositol occurs naturally in control barley leaves. To do this, the extraction and acetylation procedures were carried out as described in Sections 2.7.2 and 2.7.3 except that myo-Inositol was not added as an internal standard.

No trace of endogenous myo-Inositol could be found in barley control leaves, even at the highest sensitivity of the gas-liquid chromatograph.

- (3) Although the barley leaves had been washed with distilled water prior to extraction of internal mannitol, some mannitol may have remained on the leaf surface and would affect the calculation of internal mannitol content. To try to estimate the percentage surface contamination, control barley leaves were dipped quickly into each of the mannitol-stressing solutions and the mannitol was allowed to dry onto the leaf surface. The control barley leaves were then washed with distilled water and exposed to the

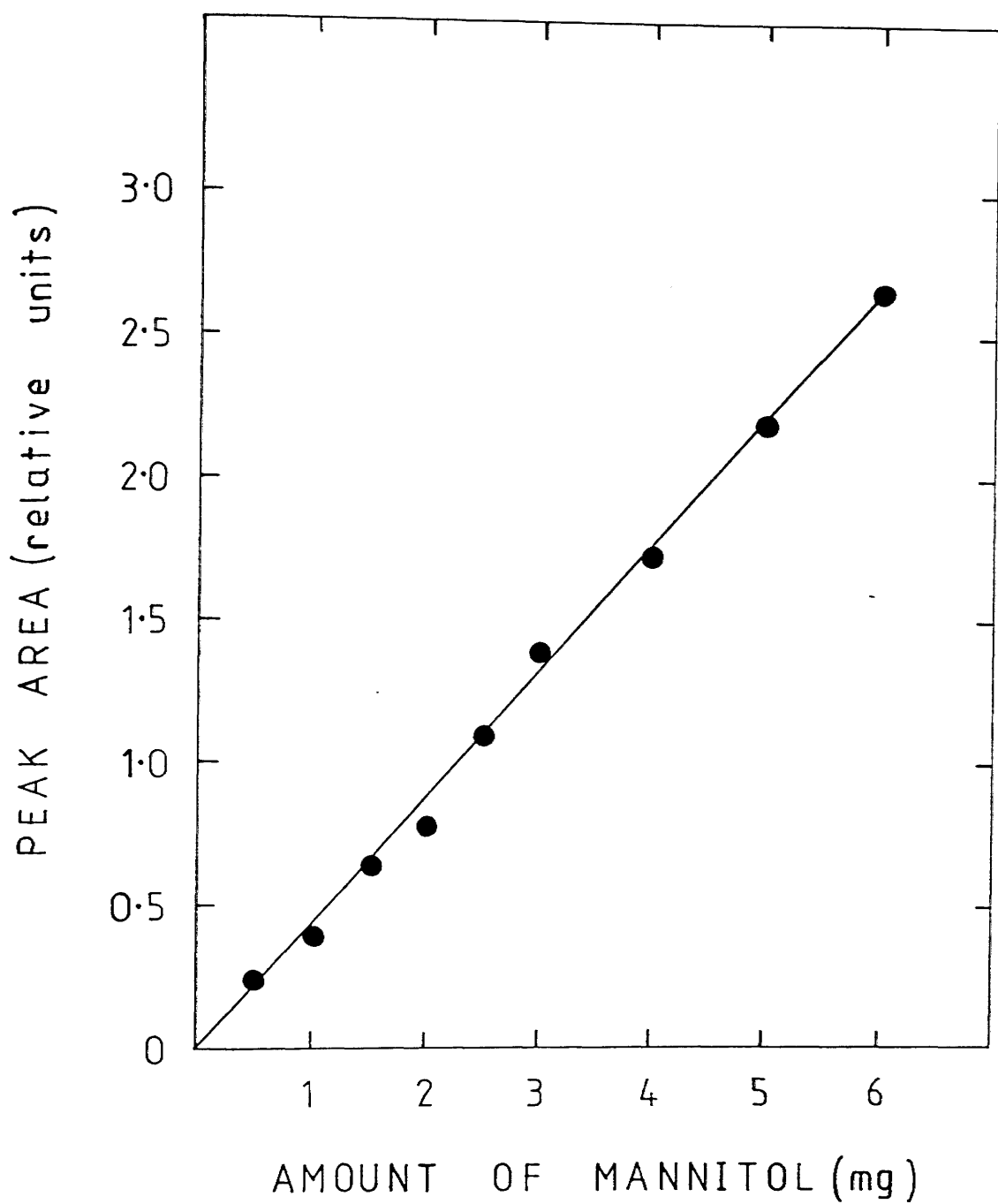


Figure 16. Response of the flame-ionisation detector of the gas-liquid chromatograph to increasing amounts of mannitol.

same extraction and acetylation procedures as described in Sections 2.7.2 and 2.7.3. Any mannitol detected above the level of mannitol found in normal control leaves was regarded as surface contamination.

2.8 Measurement of Stomata

Stomata from control and stressed barley leaf pieces were examined microscopically. Two methods were used to prepare the tissue for examination.

2.8.1 Epidermal Strips

Barley leaf pieces were submerged in liquid paraffin and then strips of upper epidermis were detached from the submerged material using forceps. The strips were transferred quickly to a slide, mounted in liquid paraffin and examined microscopically without using a cover-slip.

2.8.2 "Xantopren" Impressions

"Xantopren" (Bayer, Leverkusen, West Germany) is a light bodied silicone base precision dental impression material. A small amount was placed on a watch-glass, mixed with a drop of Elastomer Activator setting liquid (Bayer) and smeared immediately onto the upper epidermis of barley leaf pieces using a fine paintbrush. After 3 to 4 minutes the Xantopren impression had set and it was peeled off the leaf using forceps. Nail varnish was coated onto a glass slide and the impression

was pressed onto the varnish and allowed to dry for 30 minutes. The Xantopren was then removed and the impression which was left on the nail varnish was examined microscopically.

The length and width of the stomatal pore of control and stressed barley leaves were estimated using an eyepiece graticule calibrated against a stage micrometer scale.

2.9 Protoplast Isolation

Protoplasts (cells lacking cell walls) were isolated and purified by a modification of the procedure of Edwards, Robinson, Tyler and Walker (1978). Initially, a series of experiments was carried out to optimise conditions for protoplast isolation; these involved varying the pH of the solutions, the temperature of the incubation and the length of the incubation. The procedure outlined below was found to yield protoplasts of the highest photosynthetic activity.

2.9.1 Incubation of the Tissue

Barley leaf pieces were cut into segments of width 0.75mm or less using a sharp razor blade. The segments (approx. 1.5g) were placed in 20ml of enzyme medium in a 500ml conical flask and were spread evenly over the surface of the enzyme medium using a glass rod. The enzyme medium contained 30g.l^{-1} cellulase (Onozuka R-10, Yakult Honsha Co. Ltd., 1-1-19 Higashi-Shinbashi, Minato-ku, Tokyo), 5g.l^{-1} pectinase (Macerozyme R-10, Yakult Honsha Co. Ltd.), 500mM mannitol, 1mM CaCl_2 , 5% bovine serum albumin (BSA) and 5mM 2[N-

Morpholino]ethanesulfonic acid (MES) at pH 5.5. The flask was incubated for 3 hours in a 25°C water bath without shaking. During the incubation the flask was illuminated by a 150W tungsten bulb (suspended about 15cm above the top of the flask) which provided a light intensity of 16Wm^{-2} at the surface of the leaf segments. A clear dish containing a 3cm layer of water was placed between the flask and the lamp to remove excess heat.

2.9.2 Protoplast Purification

Protoplasts may become broken during incubation and isolation so that the intact protoplasts may be heavily contaminated by chloroplasts and by cell debris. However, intact protoplasts are less dense than chloroplasts and can be separated from them by flotation.

The enzyme medium was decanted from the flask and discarded since it contained very little chlorophyll. The leaf segments were then washed three times by very gentle shaking with 20ml aliquots of ice-cold wash medium (500mM mannitol, 1mM CaCl_2 and 5mM MES (pH 6.0)). The washing released the protoplasts from the leaf tissue. After each washing the protoplast suspension was filtered through a 500 μm -aperture mesh tea strainer (to retain leaf segments) and then through 200 μm -aperture nylon mesh (Henry Simon Ltd., P.O. Box 31, Stockport, England SK3 0RT) to retain released vascular strands. The filtrate was placed in six test tubes (10.5cm x 1.5cm) and centrifuged at 100 x g for 6 minutes in a Hettich EBA 3S bench-top centrifuge (Arnold R. Horwell Ltd., Maygrove Rd., West Hampstead, London NW6 2BP). After centrifugation

the supernatants were discarded and the pellets were resuspended, with very gentle shaking, in a minimal volume of ice-cold wash medium. Five ml of wash medium was then poured into each tube and each tube was mixed thoroughly by gentle inversion. The tubes were centrifuged again at 100 x g for 6 minutes, the supernatants were discarded and the pellets were resuspended in approximately 0.1ml of ice-cold sucrose suspension medium (500mM sucrose, 2mM CaCl_2 and 5mM N-Tris (hydroxymethyl)methylglycine (Tricine) at pH 7.8). Five ml of sucrose suspension medium was added to each tube; each tube was then mixed thoroughly by gentle inversion. Two ml of ice-cold sucrose density medium (400mM sucrose, 100mM mannitol, 1mM CaCl_2 and 5mM Tricine (pH 7.6)) was then layered onto the sucrose suspension medium, followed by 1ml of ice-cold mannitol density medium (500mM mannitol, 1mM CaCl_2 and 5mM Tricine (pH 7.6)). The layers were added carefully to ensure that they did not mix. After centrifugation at 200 x g for 6 minutes, the protoplasts collected in a band between the layers of sucrose density medium and mannitol density medium. All other cell debris was present in the pellet. The protoplasts were removed very carefully using a Pasteur pipette and stored in wash medium on ice in the light.

2.9.3 Determination of Protoplast Intactness

The percentage intactness of the protoplast preparations (as defined by integrity of the plasmalemma) was estimated using Evans Blue stain. This stain is only taken up by leaky, non-

living cells, making such cells easy to distinguish from green, intact cells.

One drop of Evans Blue stain (1% w/v made up in a solution containing 500mM mannitol, 1mM CaCl_2 and 5mM Tricine (pH 7.6)) was added to one drop of protoplast suspension. After 15 minutes, the percentage intactness of the protoplasts was determined microscopically.

2.10 Determination of Chlorophyll Content

The chlorophyll content of 80% acetone extracts of leaf pieces and protoplasts was determined spectrophotometrically by the method of Arnon (1949).

2.10.1 Leaf Pieces

Leaf pieces (0.1g) were ground thoroughly in 10ml of 80% (v/v) acetone in the dark using a mortar and pestle. The solution was filtered through Whatman No. 1 filter paper into a flask covered with aluminium foil. The mortar and pestle were then washed three times with 80% acetone to remove any remaining chlorophyll; after each wash the acetone was poured through the filter paper into the flask. The filter paper itself was then rinsed three times with fresh acetone and the acetone was allowed to drain into the flask. The optical density of the resultant chlorophyll solution was measured at 663 and 645nm against an 80% acetone blank in a Philips Pye Unicam SP8-500 spectrophotometer. The chlorophyll content was determined as follows.

Total chlorophyll ($\mu\text{g. ml}^{-1}$) = $(8.02 \times \text{OD}_{663}) + (20.2 \times \text{OD}_{645})$
corrected for the dilution factor.

2.10.2 Protoplasts

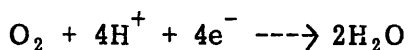
A 200 μl aliquot of protoplast suspension was added to a test tube containing 5ml of 500mM mannitol, 1mM CaCl_2 and 5mM MES (pH 6.0). The tube was covered with aluminium foil, shaken and centrifuged at 450 x g for 5 minutes in a Hettich EBA 3S bench-top centrifuge. The supernatant was discarded and the pellet was resuspended in 3ml of 80% acetone. The tube was centrifuged again at 450 x g for 5 minutes. The chlorophyll content of the resultant supernatant was estimated as before.

2.11 Simultaneous Measurement of Oxygen Evolution and Non-Modulated Chlorophyll Fluorescence from Barley Protoplasts

2.11.1 Oxygen Evolution

Oxygen evolution from barley protoplasts was measured using a modified Clark oxygen electrode (Clark, 1956) of the type described by Delieu and Walker (1972) and produced commercially by Rank Bros., Bottisham, Cambridge, England. The oxygen electrode is a device for measuring the concentration of dissolved oxygen in a solution and is sensitive enough to detect concentrations of 0.01mM (Hall and Rao, 1987). For many of the experiments described in this thesis, the electrode was adapted so that chlorophyll fluorescence could be measured at the same time as oxygen evolution.

The working principle of the oxygen electrode is based on the electrolysis of a solution of KCl. The apparatus consisted of a platinum wire sealed in plastic as the cathode and an anode of circular silver wire (Figure 17). The electrodes were linked by a bridge of saturated KCl solution and were separated from the reaction mixture in the plastic chamber by an oxygen-permeable Teflon membrane. The reaction mixture was stirred constantly with a small magnetic stirring rod. The electrodes were polarised with 0.65V; oxygen diffusing through the membrane from the reaction chamber was reduced at the platinum surface in the following reaction:-



The current which then flowed was proportional to the oxygen concentration in the reaction mixture. This current was measured by connecting the electrode to a two-channel potentiometric chart recorder (Linseis).

Calibration of the oxygen electrode consisted of determining the zero oxygen level (by addition of sodium dithionite) and the concentration of oxygen in air-saturated distilled water. Rates of oxygen evolution were expressed as $\mu\text{moles oxygen. (mg chl)}^{-1} \cdot \text{h}^{-1}$. For more details of the oxygen electrode see Allen and Holmes (1986).

2.11.2 Chlorophyll Fluorescence

Chlorophyll fluorescence from barley protoplasts was detected, via a fibre optic light guide connected to the reaction chamber of the oxygen electrode and at right angles to the exciting beam, by an EMI 9659B (extended S20) photomultiplier. The photomultiplier was protected with a Balzers 695nm

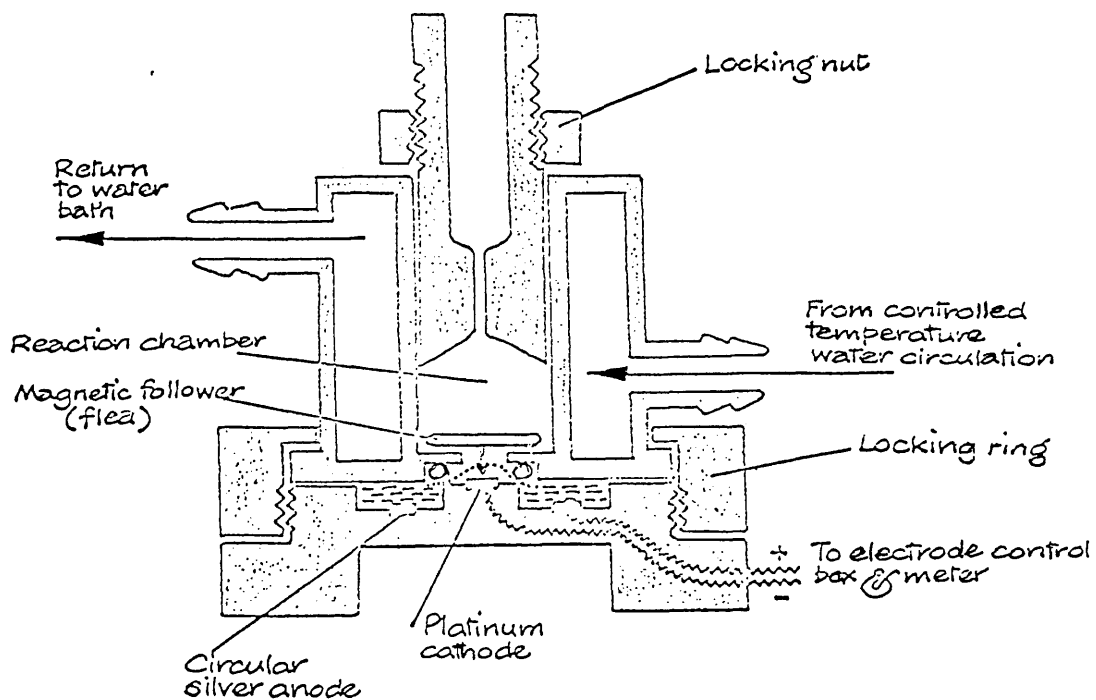


Figure 17. The oxygen electrode.

interference filter and a Schott RG 695 cut-off filter. The photocurrent was passed through a current-to-voltage converter, the output of which was fed (along with the oxygen evolution signal) directly into the two-channel chart recorder.

For all measurements of oxygen evolution and chlorophyll fluorescence from barley protoplasts, the reaction chamber was illuminated by a 150W quartz-iodine lamp powered by a Coutant stabilised DC supply. The light was filtered through Corning 4.76, Corning 4.96 and Balzers Calflex C filters to give broad-band blue light of intensity 158Wm^{-2} . The reaction chamber was kept at a constant temperature by circulating water from a temperature-controlled water bath (25°C). Protoplasts were assayed in a medium containing 500mM mannitol, 1mM CaCl_2 , 5mM Tricine (pH 7.6) and 5mM KHCO_3 . To prevent damage to the protoplasts, the magnetic stirring speed of the oxygen electrode was set at the slowest speed which would still maintain a stable response.

2.12 Simultaneous Measurement of Oxygen Evolution and Non-Modulated Chlorophyll Fluorescence from Barley Leaves

Simultaneous measurement of oxygen evolution and non-modulated chlorophyll fluorescence from barley leaf pieces was carried out using a Hansatech LD2 Leaf Disc Electrode Unit (Hansatech Ltd., Paxman Rd., Hardwick Industrial Estate, King's Lynn, Norfolk, England).

2.12.1 The Oxygen Electrode

The oxygen detector was a disc located in the lower half of

the middle section of the apparatus (Figure 18). This disc was a conventional Clark-type electrode (Delieu and Walker, 1972) which worked on the same principle as the oxygen electrode described previously (Section 2.11.1) except that the sample was in the gas phase rather than in solution. Rates of oxygen evolution were expressed as $\mu\text{moles oxygen} \cdot (\text{mg chl})^{-1} \cdot \text{h}^{-1}$.

2.12.2 The Leaf Chamber

The leaf chamber lay on top of the electrode in such a way that the platinum cathode was exposed to the atmosphere in the leaf chamber through a small hole in the floor of the chamber. The leaf chamber and the electrode disc were both encased in massive anodised aluminium through which water from a thermostatically-controlled water bath was passed in order to maintain a steady temperature. The chamber itself was cylindrical (4ml internal volume when empty) and accommodated a leaf disc of 10cm^2 area or smaller pieces of leaf carried on a perforated stainless steel disc with an unperforated centre, which prevented light falling directly onto the cathode. The upper section of the chamber was the perspex floor of the upper water jacket, through which the chamber was illuminated. When the chamber was closed, the upper section was fixed in position by two clips. O-rings were used to give an air-tight seal. Two taps, fitted to the outside of the leaf chamber, enabled the chamber to be connected to the external atmosphere. These taps were used in the calibration procedure when the oxygen partial pressure was altered by inserting or removing a known volume (1ml) of air.

To prevent the carbon dioxide content of the leaf chamber

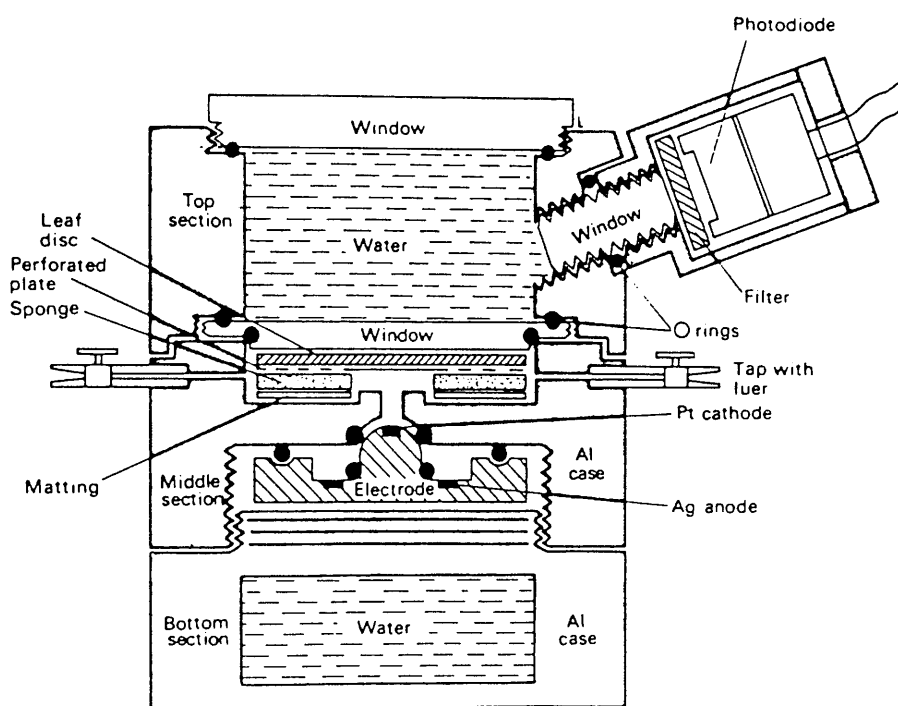


Figure 18. Schematic diagram of the gas phase oxygen electrode and chlorophyll fluorescence probe (adapted from Delieu and Walker (1983)).

becoming exhausted, potassium hydrogen carbonate buffer was used as a source of carbon dioxide. The buffer (1ml of a 1M solution) was carried on capillary matting to facilitate rapid equilibration. The leaf tissue was protected from the alkaline buffer by a second stainless steel disc and a foam rubber disc which were also inserted into the chamber (Figure 18). The foam rubber disc had an added advantage in that, when compressed, it acted like a spring and pressed the leaf tissue lightly against the temperature-controlled roof of the chamber. A probe, fitted with its own perspex window, was inserted into the chamber at an angle of 40° and allowed chlorophyll fluorescence to reach a photodiode (U.D.T. 500 Photo-op photodiode) shielded by a Wratten 88A filter. The chlorophyll fluorescence signal was amplified within the probe, from which it was passed (along with the oxygen evolution signal) to a two-channel potentiometric chart recorder (Linseis).

For all measurements of oxygen evolution and non-modulated chlorophyll fluorescence from barley leaf pieces (0.1g), blue actinic light was provided (via Corning 4.76, Corning 4.96 and Balzers Calflex C filters placed on top of the perspex window of the leaf chamber) by a 150W quartz-iodine lamp powered by a Coutant stabilised DC supply. Unless otherwise stated, the temperature of the circulating water was 25°C and the light intensity was 158Wm^{-2} .

2.13 Measurement of Low Temperature Chlorophyll Fluorescence from Barley Leaves

Measurement of chlorophyll fluorescence from barley leaf

pieces frozen to 77K was carried out in collaboration with Prof. N.R. Baker at the Biology Department, University of Essex.

Barley leaf pieces were placed in a metal sample holder which was attached to a metal tube containing a perspex light-mixing rod. The metal holder and light-mixing rod were plunged into a Dewar flask containing liquid nitrogen and held there for 10 minutes to ensure that a temperature of 77K had been reached. Bifurcated fibre optic light guides were used to transmit the excitation radiation to, and the chlorophyll fluorescence from, the leaf surface. A slide projector was used to illuminate the leaves with 27Wm^{-2} of 440nm irradiation. Measurement of chlorophyll fluorescence emission from 650-800nm was made through a scanning high radiance monochromator (Applied Photophysics) with entry and exit slits of 1.25mm and 5nm respectively. Chlorophyll fluorescence was detected by a Hamamatsu R446 photomultiplier and passed directly to a chart recorder.

2.14 Measurement of Modulated Chlorophyll Fluorescence from Barley Leaves

2.14.1 Measurement of the Redox State of Q During Kautsky Transients : "Light Doubling"

A modification of the "light doubling" technique of Bradbury and Baker (1981) was used to determine what proportion of Q (Section 1.5.2) was in the oxidised state during the Kautsky transients (Section 1.8.1) of barley leaf pieces; using this technique a second actinic light of high intensity was applied to the system at appropriate intervals during the

transient and the resultant rapid increase in the chlorophyll fluorescence signal was used to calculate the proportion of oxidised Q.

The experimental set-up used to measure light doubling from barley leaf pieces is shown in Figure 19. For convenience, the LD2 Leaf Disc Electrode Unit described in Section 2.12 was positioned on its side. Light from a 150W quartz-iodine lamp powered by a Coutant stabilised DC supply was focused by means of a biconvex lens through the slit of a Brookdeal 9479 light chopper set at 189Hz. The modulated light was passed through a biconvex lens and Corning filters (4.96 and 4.76) which were placed on top of the perspex window of the leaf disc unit. The lens focused the blue-green modulated light onto a section of leaf 0.7cm in diameter, giving an irradiance of 21Wm^{-2} . The rest of the leaf surface was covered with a black disc. Modulated chlorophyll fluorescence was detected using a photodiode (U.D.T. 500 Photo-op photodiode) shielded by a Wratten 88A filter. The signal was transduced by a Brookdeal lock-in amplifier and fed into a chart recorder (Linseis).

A second blue-green light source similar to the first was used to provide non-modulated light. A biconvex lens positioned above a porthole in the side of the leaf disc unit was used to focus this non-modulated light through a Prontor-Press shutter onto the leaf surface. The shutter was operated manually using a cable release to give 1 second light pulses spaced 10 seconds apart. The intensity of the light pulses at the leaf surface was 12.5Wm^{-2} . The temperature of the water circulating through the leaf disc unit was 25°C .

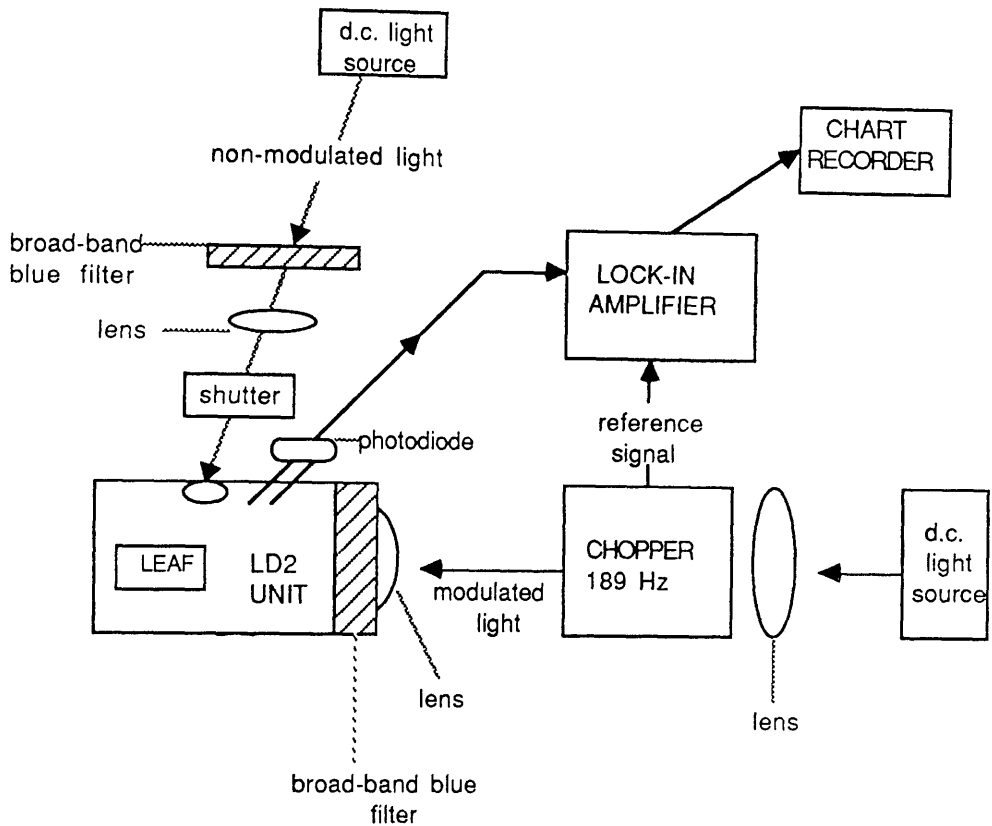


Figure 19. The experimental set-up used to measure light doubling from barley leaf pieces. Exciting light of irradiance 21Wm^{-2} was transmitted by Corning 4.96 and Corning 4.76 filters, and was modulated at 189Hz. Non-modulated light pulses, of irradiance 12.5Wm^{-2} , were transmitted by Corning 4.96 and Corning 4.76 filters. The modulated chlorophyll fluorescence signal, transmitted by a Wratten 88A filter, was detected by a photodiode coupled to a lock-in amplifier.

2.14.2 State 1 - State 2 Transitions

To measure State 1 - State 2 transitions (Section 1.5.5) in barley leaf pieces, the experimental set-up was the same as that used for light doubling (Section 2.14.1) except that no shutter was used, the non-modulated light was transmitted through a Schott RG 715 long-pass filter, and the photodiode was shielded by a Balzers 695nm interference filter. The light intensity of the non-modulated light at the leaf surface was 7Wm^{-2} .

2.15 Calculation of Standard Error of the Mean

Values for standard error of the mean (S.E.M.) were calculated as below:

$$\text{S.E.M.} = \frac{s}{\sqrt{n}}$$

where,

$$s = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

x is the individual value of each observation

n is the number of observations

SALT STRESS

3.1 Selection of a Growth Support in which to Stress Barley Leaves

In order to stress barley leaves, a growth support was required which was chemically inert and had a known nutrient composition which could easily be controlled. At the same time, however, the oxygen evolution rate of leaves grown in such a growth support had to be comparable with the high rates regularly observed from leaves grown in potting compost (i.e. $\sim 220 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{h}^{-1}$).

The oxygen evolution rates of barley leaves grown in perlite (expanded volcanic lava), vermiculite (expanded mica) and sand were compared with those of leaves grown in potting compost (Figure 20). In all four growth supports, the rate of oxygen evolution rose with age initially, reaching a maximum at 8 days before falling sharply at 10 days. Apart from potting compost, perlite supported the highest oxygen evolution rates. Consequently, perlite was chosen as a growth support in which to stress barley leaves.

3.2 The Growth, Water Relations and Ionic Content of Salt-Stressed Barley Leaves

3.2.1 The Effect of Increasing the External [NaCl] on the Height of the Primary Leaves of Barley

To determine whether salt stress had any effect on the growth of barley, 4 day old seedlings were stressed with NaCl ranging in concentration from 0 to 600mM. The heights of the

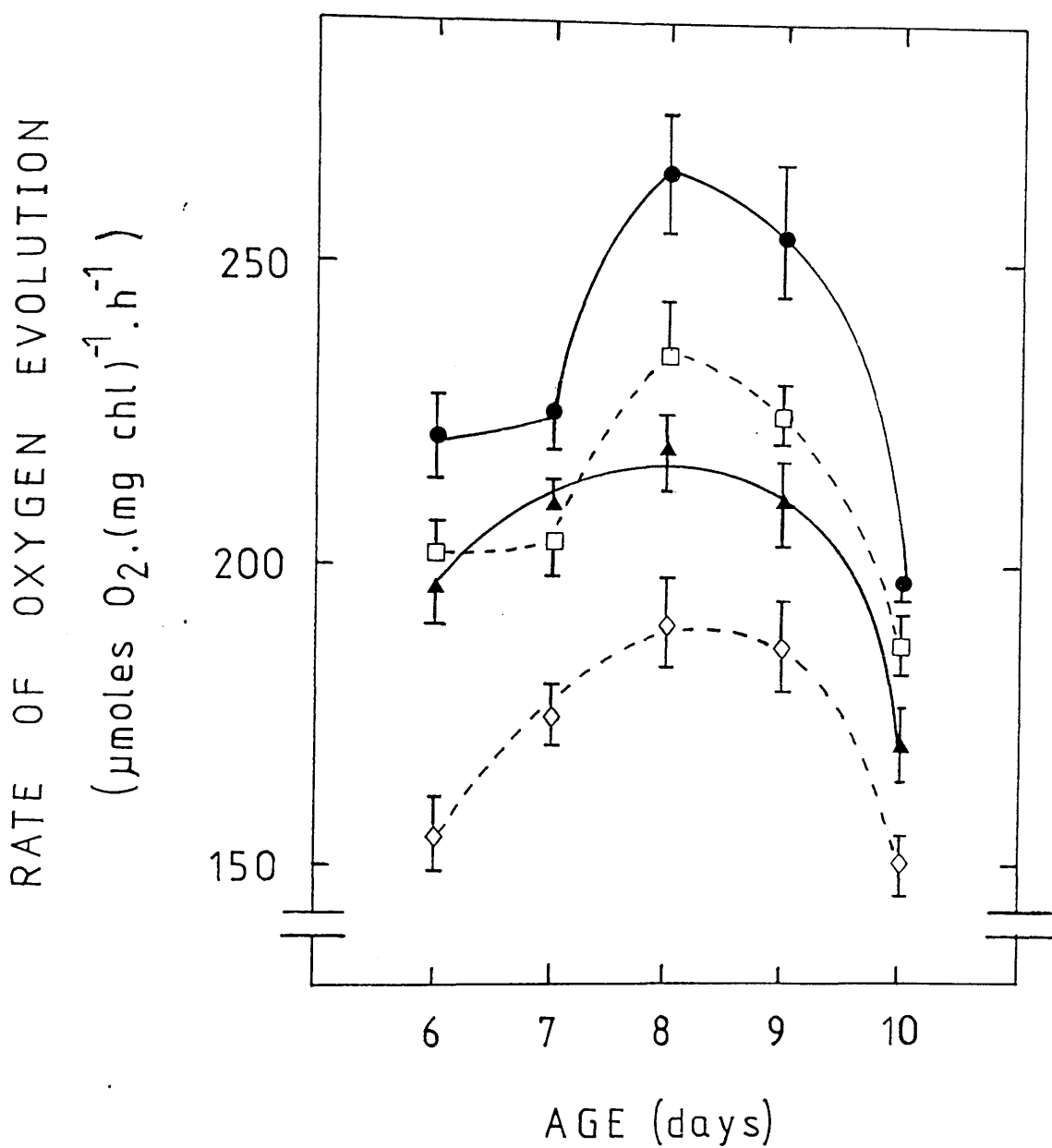


Figure 20. Steady-state oxygen evolution rates of barley leaf pieces grown in potting compost (●—●), perlite (□----□), vermiculite (▲—▲) and sand (◇----◇). The results indicate the mean \pm S.E.M. of 3 determinations.

primary leaves of 7 and 14 day old stressed plants were then measured and compared with those of control plants (Figure 21). Figure 21 also shows the osmotic potentials of the stressing solutions, as measured by freezing point depression using the Knauer apparatus (Section 2.4).

At 7 days old, the height of the primary leaves was almost unaffected by NaCl concentrations up to 200mM; above 200mM the height decreased steadily as the salinity was increased. After a further period of 7 days, the difference in the height of control and salt-stressed leaves was much more pronounced. Leaves stressed with 600mM NaCl showed almost no growth after the first period of 7 days.

In all subsequent salt-stress experiments, 4 day old barley leaves were stressed by nutrient solution supplemented with 0, 250, 400 or 600mM NaCl. These salt concentrations were chosen because, as is shown in Figure 21, they seem to expose the leaves to a range of salt stress. In addition, many of these experiments were carried out over a period of three days to determine whether increased exposure of the leaf to NaCl resulted in a more pronounced effect. The experiments were carried out when the leaves were 7, 8 and 9 days old because, at these ages, the control leaves had the highest rates of oxygen evolution (Figure 20).

3.2.2 The Effect of Increasing the External [NaCl] on the Water Potential of Barley Leaves

As noted previously (Section 1.3.1.1) osmotic stress normally leads to a loss of water from plants. To determine the extent to which barley leaves lose water when exposed to increasing

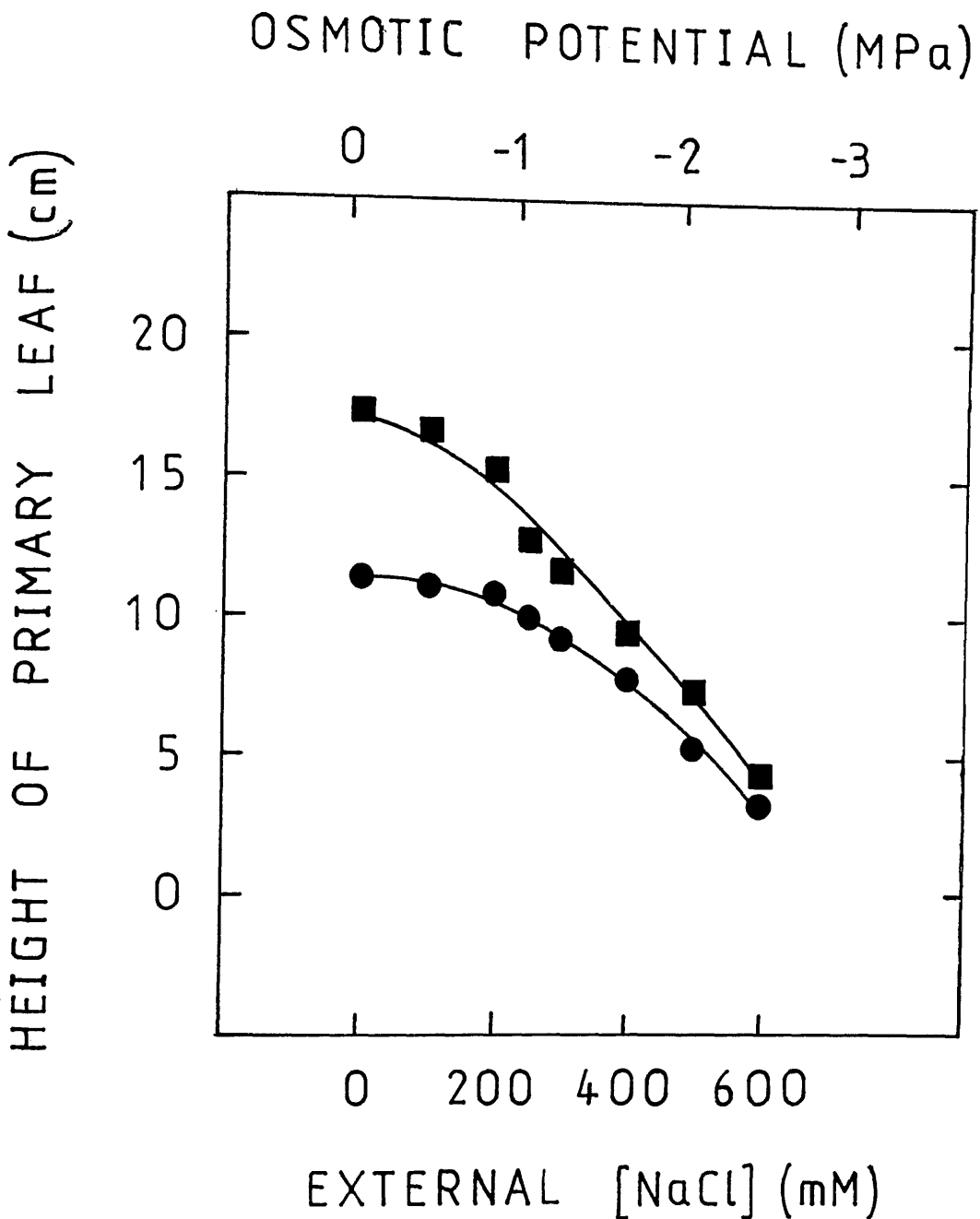


Figure 21. The effect of increasing the external [NaCl] on the height of the primary leaves of barley measured after 7 days (● — ●) and 14 days (■ — ■). Each point represents the mean of 20 determinations. Standard error bars were within the symbol size.

concentrations of NaCl, the water potentials of 7, 8 and 9 day old control and salt-stressed leaves were compared using the pressure chamber described in Section 2.5. Figure 22 shows these results.

Increasing the external [NaCl] resulted in a fall in the water potential of 7 day old barley leaves. In 8 day old leaves, the water potential fell more steeply but rose slightly at 600mM NaCl. In 9 day old leaves, the water potential fell very sharply at 250mM NaCl, only to rise as the external [NaCl] was increased above 250mM. The results indicate that barley was being exposed to osmotic stress and that the effect of this stress became more pronounced with leaf age.

3.2.3 The Effect of Increasing the External [NaCl] on the Internal $[\text{Na}^+]$ of Barley Leaf Pieces

The internal $[\text{Na}^+]$ concentrations of 7, 8 and 9 day old control and salt-stressed barley leaf pieces were estimated using the flame photometer described in Section 2.6. The results are shown in Figure 23.

In each of the three sets of leaf pieces, the internal $[\text{Na}^+]$ increased as the external salinity was increased. In addition, the longer the leaves were exposed to salt the higher the internal $[\text{Na}^+]$. Seven and eight day old barley leaf pieces showed a fairly small rise in internal $[\text{Na}^+]$ between external NaCl concentrations of 0 to 400mM. In 8 day old leaf pieces, the internal $[\text{Na}^+]$ showed a large rise at an external [NaCl] of 600mM. In 9 day old leaf pieces, this large rise in internal $[\text{Na}^+]$ occurred at a lower external [NaCl] (400mM).

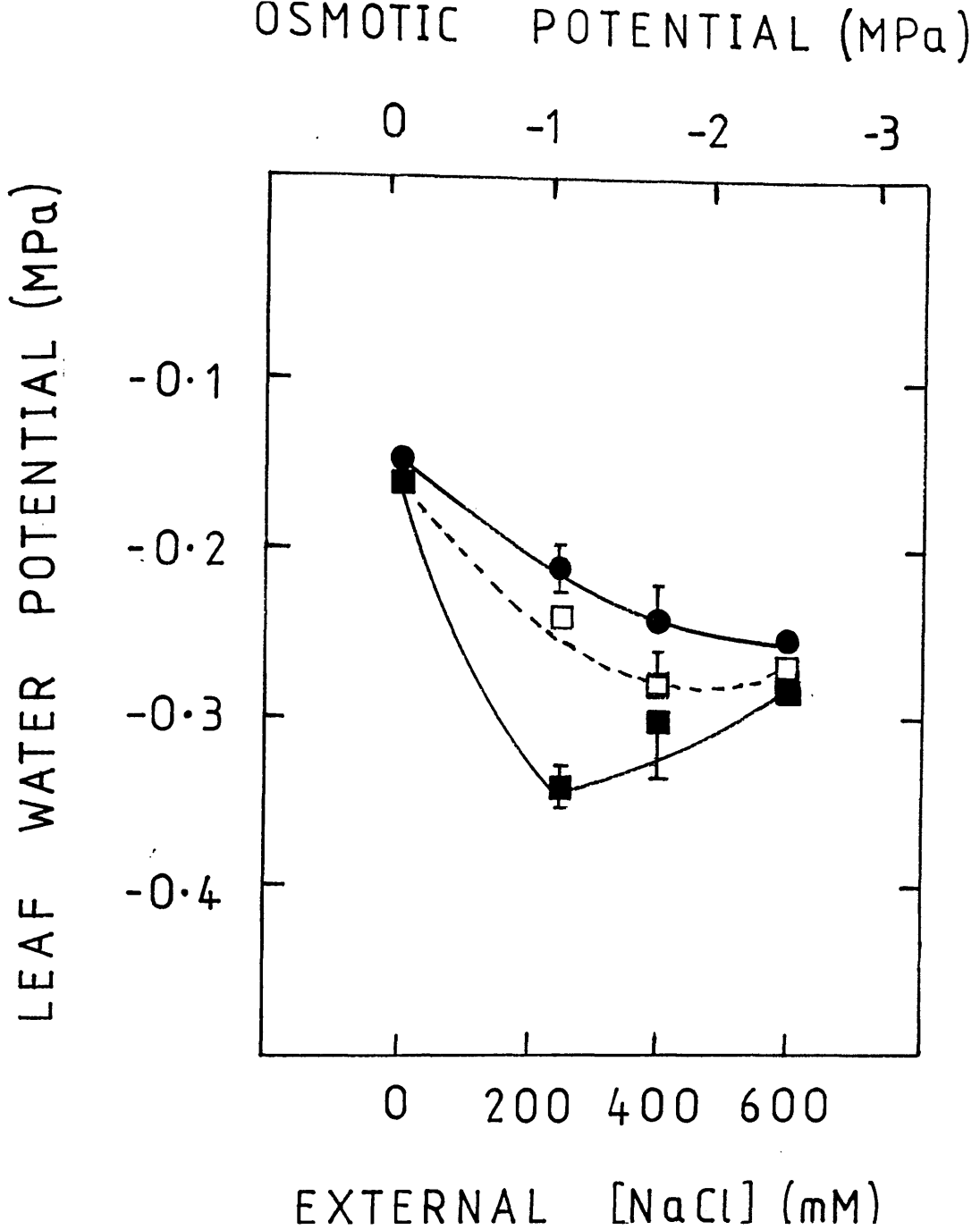


Figure 22. The effect of increasing the external [NaCl] on the water potential of 7 (●—●), 8 (□---□) and 9 (■—■) day old barley leaves. Each point represents the mean \pm S.E.M. of 10 determinations. Where standard error bars are not shown, errors were within the symbol size.

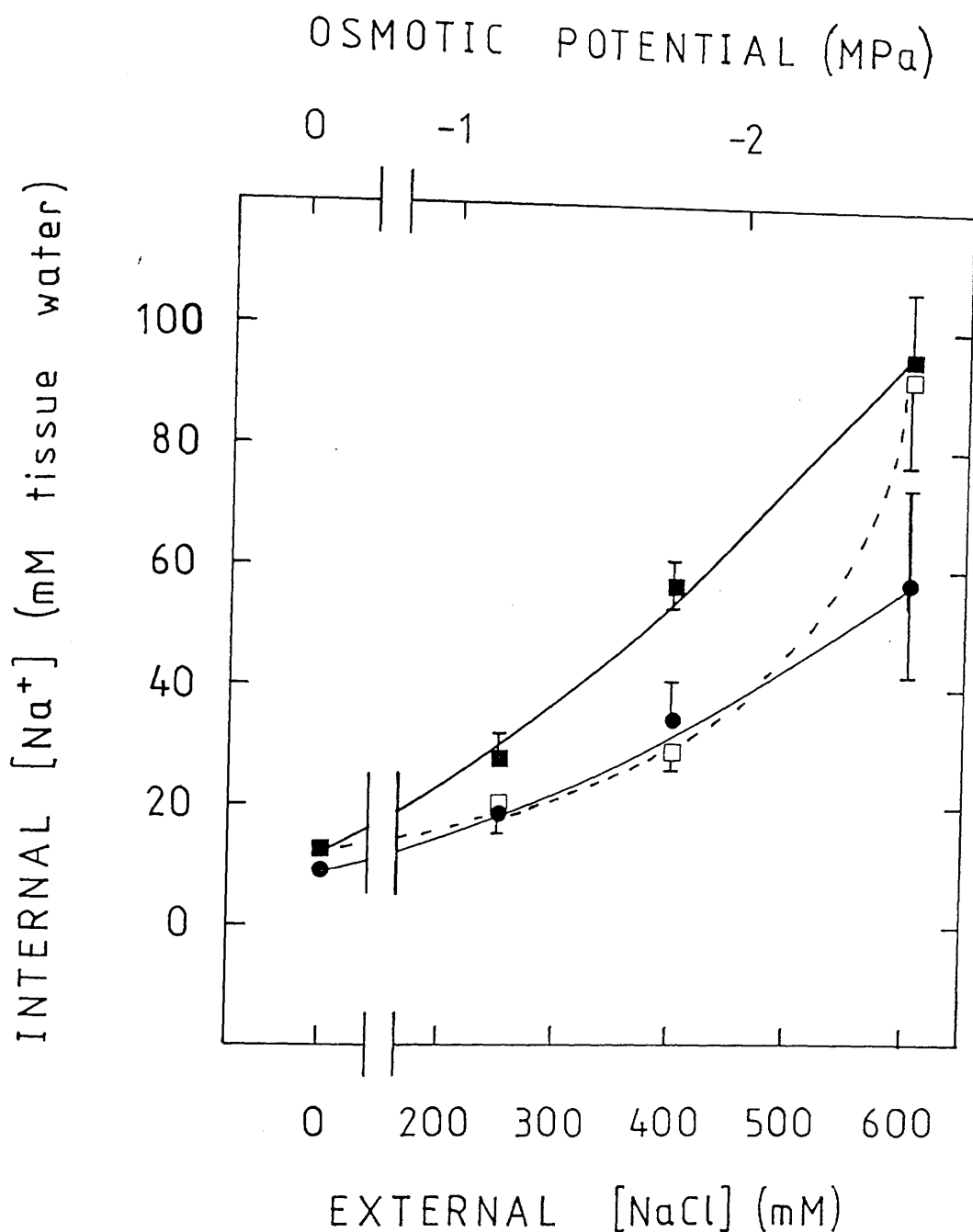


Figure 23. The effect of increasing the external $[NaCl]$ on the internal $[Na^+]$ of 7 (●—●), 8 (□—□) and 9 (■—■) day old barley leaf pieces. Each point represents the mean \pm S.E.M. of 3 determinations. Where standard error bars are not shown, errors were within the symbol size.

3.2.4 Discussion

The most obvious morphological difference between control and salt-stressed barley was the reduced height of the primary leaves of the salt-stressed plants. This agrees with results obtained by Kumar, Singh and Sharma (1981) for barley and by Epstein (1962) and Rai (1977) for other plant species. The reduced growth of the salt-stressed leaves was probably a consequence of the lower water potentials of these leaves compared with the control leaves. Loss of water as a result of osmotic stress would lead to a loss of turgor which would, in turn, lead to an inhibition of growth. In general, it would be expected that the higher the external [NaCl] the lower the leaf water potential and so the greater the inhibition of growth.

However, although the height of 7, 8 and 9 day old barley leaves did indeed continue to fall as the external salinity was increased from 200 to 600mM NaCl, a continual fall in leaf water potential with increasing salinity was seen only in 7 day old leaves. At 8 days old, the water potential of leaves stressed by 600mM NaCl was higher (i.e. less negative) than that of leaves stressed by 400mM NaCl. This was also the case in 9 day old leaves but, in addition, the water potential of leaves stressed by 400mM NaCl was higher than that of leaves stressed by 250mM NaCl. A possible explanation for this decline and subsequent rise in the leaf water potential involves the concentration of Na^+ ions inside the leaves. Taking the example of 8 day old barley leaves, if no or few Na^+ ions entered those leaves which had been stressed by the lower NaCl concentrations (250 or 400mM), then these leaves would lose water to the external environment. The leaf water potential would fall below that of control leaves

until an equilibrium was achieved with the external environment. If, however, the roots became damaged when leaves were stressed by the highest NaCl concentration (600mM) then Na^+ ions would flood into these leaves and give rise to a much higher internal $[\text{Na}^+]$. This high $[\text{Na}^+]$ would be expected to help protect these leaves from water loss (by lowering the difference in water potential between the leaves and their external environment), resulting in a higher leaf water potential than that of leaves stressed by 250 or 400mM NaCl.

This explanation appears to be supported by the results of the experiment to determine the internal $[\text{Na}^+]$ of salt-stressed leaves. Those leaves which had a higher water potential than leaves stressed by lower NaCl concentrations also had a much higher internal $[\text{Na}^+]$.

3.3 Simultaneous Measurement of Oxygen Evolution and Chlorophyll Fluorescence During the Induction Period of Photosynthesis in Barley Leaf Pieces

Before investigating the effect of salt stress on oxygen evolution and chlorophyll fluorescence during the induction period of photosynthesis, experiments were carried out to optimise conditions for simultaneously measuring these two phenomena using the LD2 Leaf Disc Electrode.

3.3.1 Varying the Length of the Dark Period Prior to Illumination

The simultaneous oxygen evolution and chlorophyll fluorescence induction curves of barley leaf pieces illuminated after dark periods of 3, 10 and 30 minutes are shown in Figure 24.

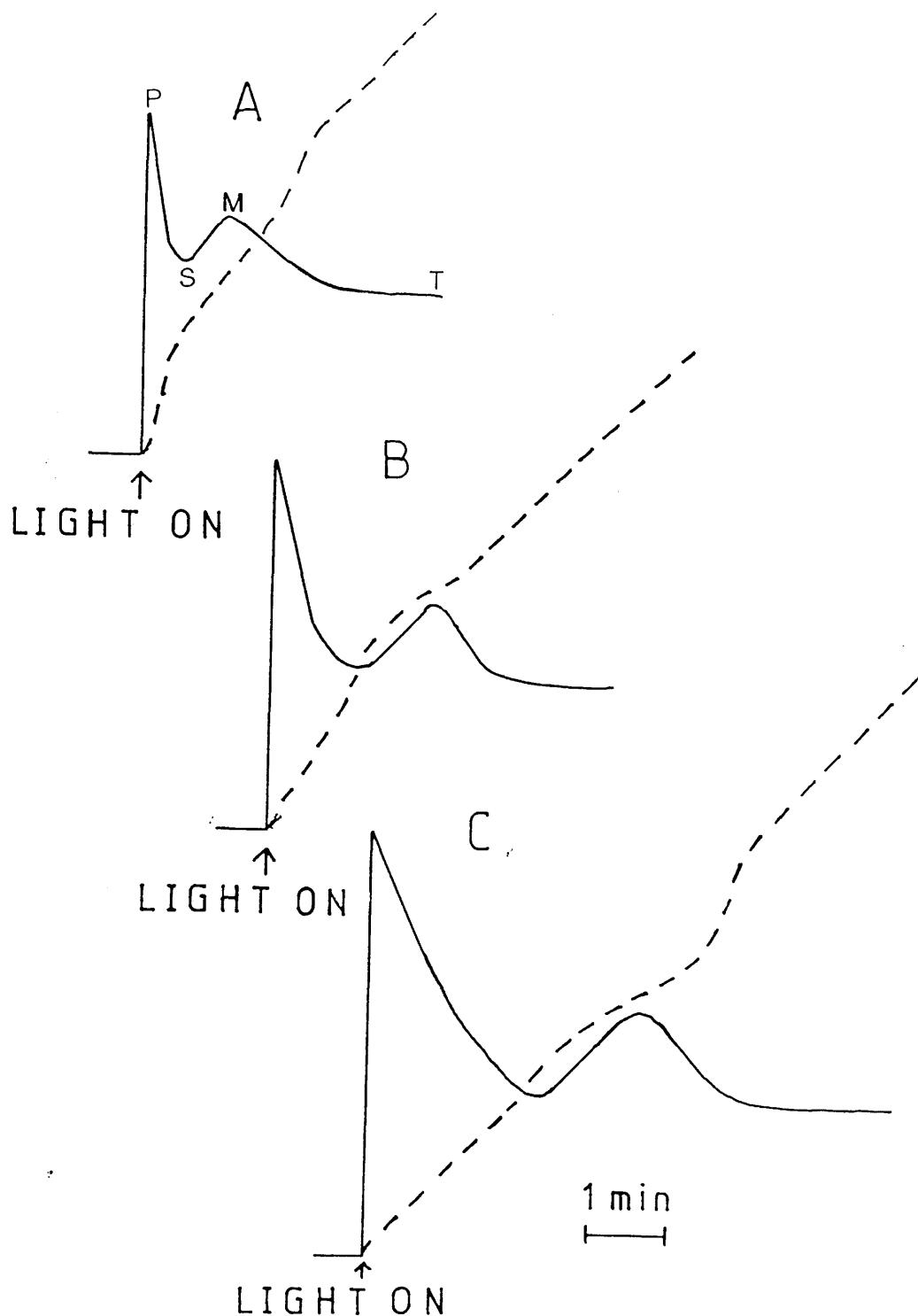


Figure 24. Simultaneous chlorophyll fluorescence (solid line) and rate of oxygen evolution (dashed line) during the induction period of barley leaf pieces following dark - adaptation periods of A) 3 minutes; B) 10 minutes and C) 30 minutes.

3.3.1.1 Effect on Oxygen Evolution

Oxygen evolution during induction was shown to consist of three phases. The first phase was a burst in the rate of oxygen evolution followed immediately by a decrease in rate. The second phase was a rise in the rate of oxygen evolution, and the third phase was oscillations in the rate of oxygen evolution, after which it eventually reached its steady-state rate. The effect of increasing the dark period prior to illumination was to decrease the initial burst in oxygen evolution and increase the time taken for oxygen evolution to reach a steady-state. The steady-state rate itself was unaffected by the length of dark pretreatment.

3.3.1.2 Effect on Chlorophyll Fluorescence

The effects of increasing the dark period prior to illumination were to increase the chlorophyll fluorescence level at P, increase the magnitude of the S-M transient and increase the time required for chlorophyll fluorescence to attain a steady-state. The steady-state level of chlorophyll fluorescence was unaffected by the length of the dark pretreatment.

In addition, the chlorophyll fluorescence and oxygen evolution signals were seen to be anti-parallel and slightly phase-shifted with chlorophyll fluorescence preceding alteration in the rate of oxygen evolution; the initial burst in oxygen evolution was associated with the pronounced decline in chlorophyll fluorescence PS, while the following decrease in oxygen evolution was correlated with the rise in chlorophyll fluorescence SM.

3.3.2 Varying the Light Intensity

The effect of increasing the light intensity on the steady-state rate of oxygen evolution and on the chlorophyll fluorescence induction curve following a dark period of 10 minutes is shown in Figures 25 and 26.

3.3.2.1 Effect on Oxygen Evolution

As the light intensity was increased, the rate of steady-state oxygen evolution increased and was close to saturation at an intensity of 158Wm^{-2} .

3.3.2.2 Effect on Chlorophyll Fluorescence

Although the oscillations were slightly more frequent in high light, the major effect of increasing the light intensity was to increase the amplitude of the chlorophyll fluorescence signal i.e. the height of P, M and T all increased. The rate of quenching from P to S was largely independent of light intensity.

3.3.3 Varying the Temperature

The effect of temperature on oxygen evolution during the steady-state is shown in Table I. Chlorophyll fluorescence induction curves at 10°C, 15°C, 20°C and 25°C following a dark period of 10 minutes are shown in Figure 27.

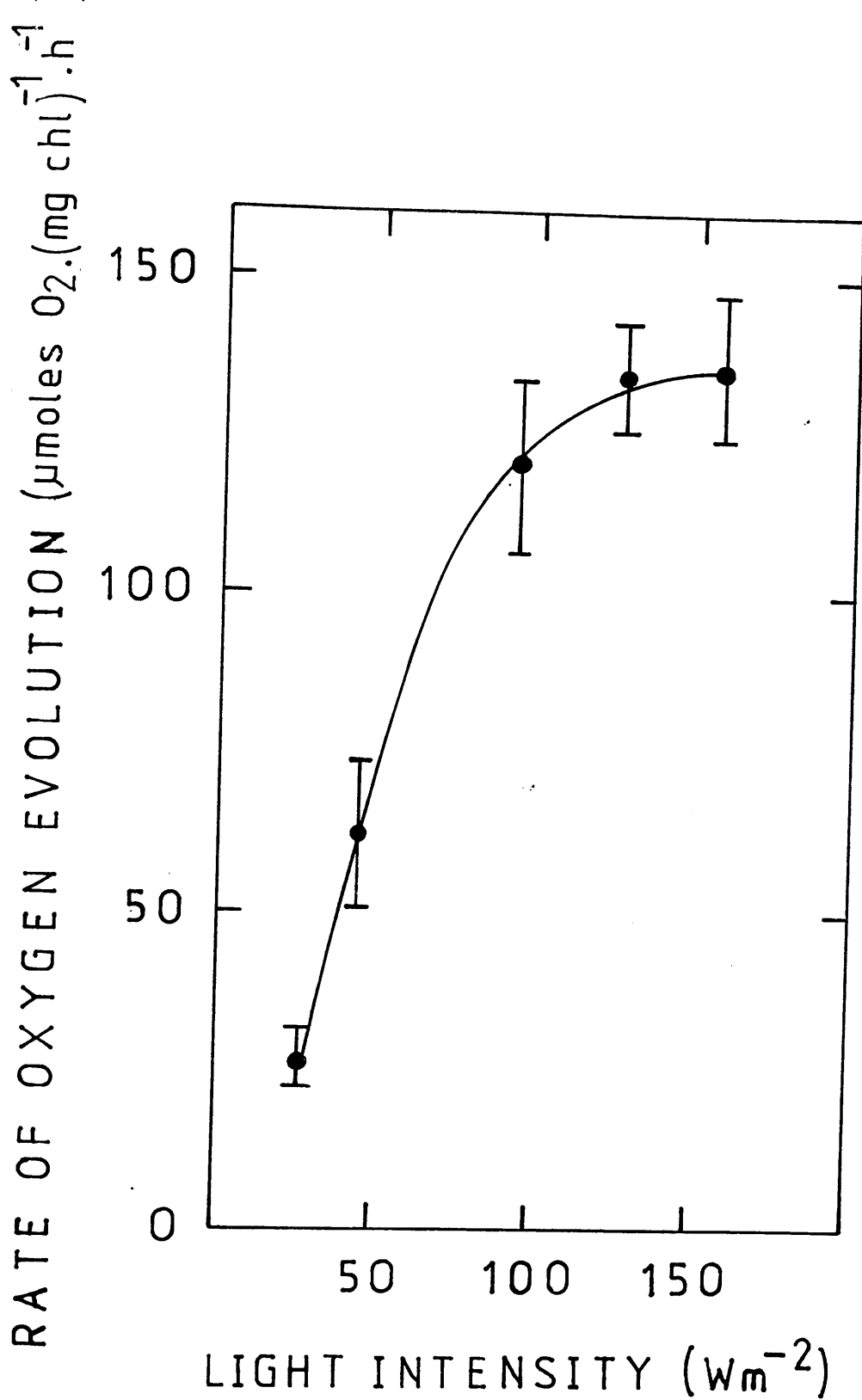


Figure 25. The effect of light intensity on the steady-state rate of oxygen evolution by barley leaf pieces. The leaf pieces were illuminated with broad-band blue light (Corning 4.96, Corning 4.76 and Balzers Calflex C filters). Light intensity was adjusted by means of neutral density filters. Results indicate the mean \pm S.E.M. of 3 determinations.

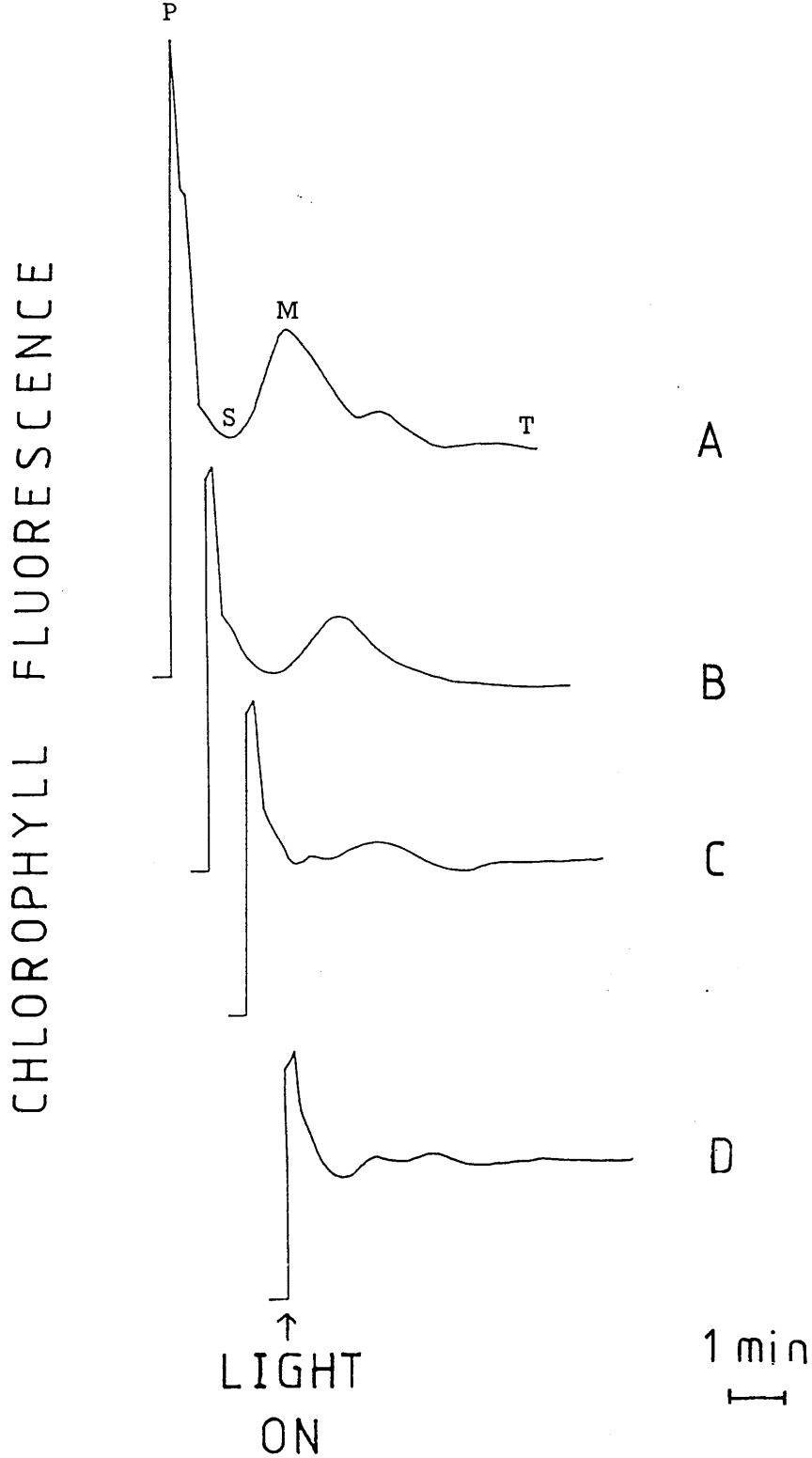


Figure 26. The effect of light intensity on chlorophyll fluorescence from barley leaf pieces following a dark period of 10 minutes. A) 159Wm^{-2} ; B) 127Wm^{-2} ; C) 110Wm^{-2} ; D) 94Wm^{-2} . The leaf pieces were illuminated with broad-band blue light (Corning 4.96, Corning 4.76 and Balzers Calflex C filters) and the photodiode was protected by a Wratten 88A filter. Light intensity was adjusted by means of neutral density filters.

T A B L E I

The Effect of Varying the Temperature on the Steady-State Rate of Oxygen Evolution by Barley Leaf Pieces

The leaf pieces were illuminated by broad-band blue light (Corning 4.96, Corning 4.76 and Balzers Calflex C filters). The results indicate the mean \pm S.E.M. for 3 determinations.

Temp. (°C)	Oxygen evolution rate (μ moles O ₂ .(mg chl) ⁻¹ . h ⁻¹)
25	216 \pm 12.1
20	126 \pm 8.1
15	89 \pm 14.7
10	37 \pm 4.1

CHLOROPHYLL FLUORESCENCE

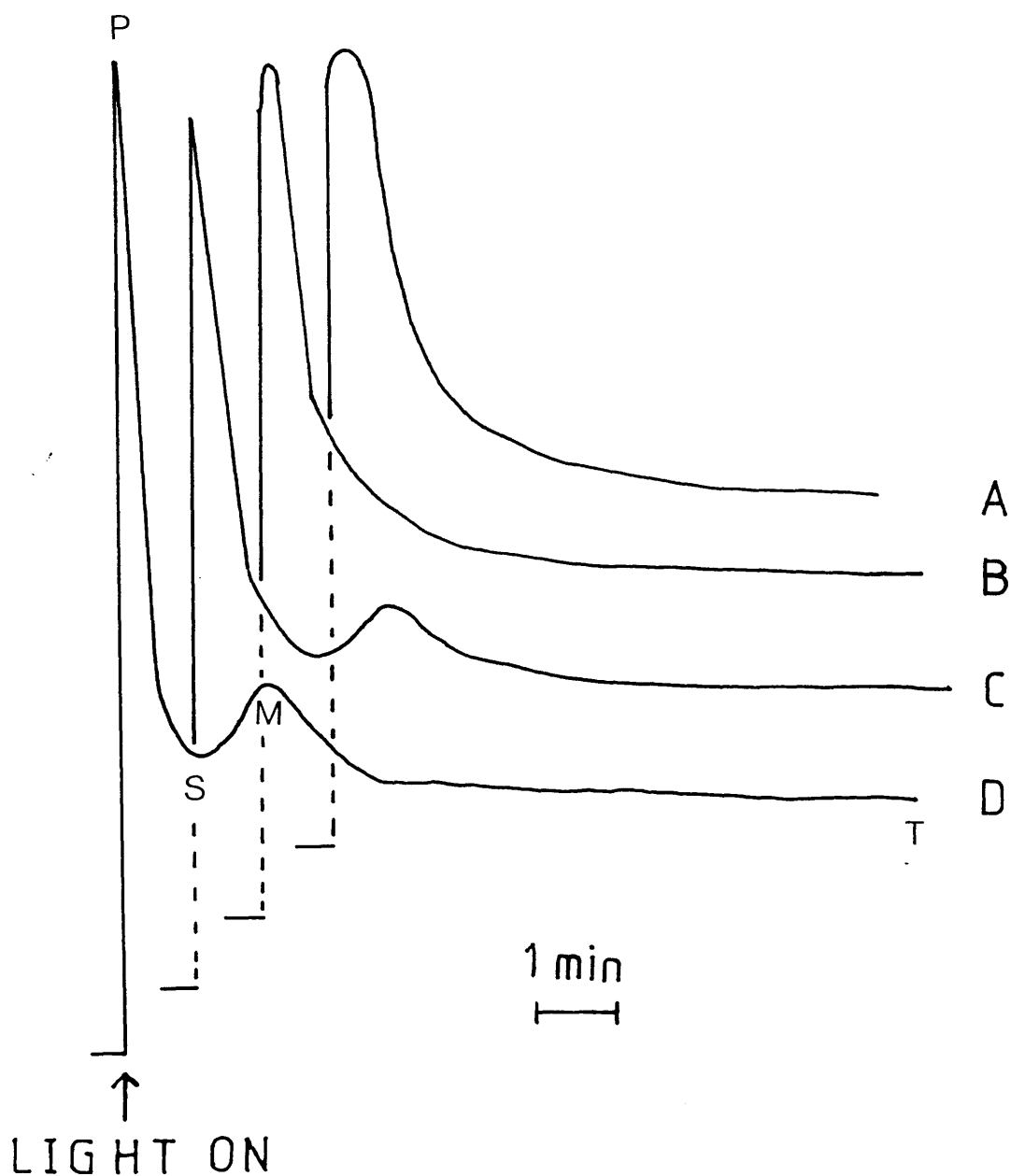


Figure 27. The effect of temperature on the chlorophyll fluorescence induction curves of barley leaf pieces following a dark period of 10 minutes. A) 10°C; B) 15°C; C) 20°C; D) 25°C. The leaf pieces were illuminated with broad-band blue light (Corning 4.96, Corning 4.76 and Balzers Calflex C filters) and the photodiode was protected by a Wratten 88A filter.

3.3.3.1 Effect on Oxygen Evolution

The rate of steady-state oxygen evolution decreased as the temperature decreased.

3.3.3.2 Effect on Chlorophyll Fluorescence

As the temperature decreased, P was largely unchanged but the S-M transient became progressively smaller and chlorophyll fluorescence quenched to an increasingly higher steady-state level. In addition, the period of the oscillation increased i.e. the time to reach the top of the S-M transient increased.

3.3.4 Discussion

Varying the length of the dark period prior to illumination of barley leaf pieces had the same effect on the induction period as observed previously in maize leaves (Ireland, Long and Baker, 1984). The results support the hypothesis that induction is due to light activation of enzymes in the RPP pathway or to build-up of intermediates of the RPP pathway or both; during a short dark period, only a small amount of both enzyme deactivation and dissipation of substrates can occur so that, upon illumination, a shorter time will be required to re-activate enzymes or build-up any depleted intermediates. Consequently, the time required for both oxygen evolution and chlorophyll fluorescence to reach a steady-state will be shorter than that observed following a long period of dark adaptation.

The effect of light intensity and temperature on the induction period also supports the above hypothesis and is in accord with other results (Walker, 1976; Quick and Horton, 1984a). Induction was shown to be relatively independent of light intensity, whereas low temperature increased the period of the oscillation. This suggests that induction is more related to the temperature-dependent enzymic reactions of the RPP pathway than to photochemical processes such as electron transport.

The anti-parallel relationship observed between oxygen evolution and chlorophyll fluorescence during the induction period has been noted previously in barley protoplasts (Quick and Horton, 1984a) and a similar anti-parallel relationship has recently been shown to occur between chlorophyll fluorescence and CO₂ assimilation in maize leaf pieces (Ireland et al., 1984). Since chlorophyll fluorescence competes with photochemistry for excitation energy, such anti-parallel relationships are not unexpected. The phase shift observed between the oxygen evolution and chlorophyll fluorescence signals has also been seen in barley protoplasts (Quick and Horton, 1984a) and leaves (Walker, Sivak, Prinsley and Cheesbrough, 1983) but has not yet been explained fully. The initial burst in the rate of oxygen evolution followed immediately by a decrease is thought to reflect the fast rate of electron transport to NADP pools present at the end of the dark period and the subsequent slowing down of electron transport as these oxidant pools become exhausted (Horton, 1983).

In the following experiments to investigate the effect of salt on oxygen evolution and chlorophyll fluorescence during the induction period, Kautsky transients were induced at a temperature of 25°C by a light intensity of 158Wm⁻² following a dark interval of 10 minutes. Oxygen evolution was measured

simultaneously and the rate of steady-state oxygen evolution was measured 7 minutes after illumination i.e. during the T phase.

3.4 The Effect of Increasing the External [NaCl] on the Oxygen Evolution Rate of Barley Leaf Pieces

Before using the Leaf Disc Electrode to measure the oxygen evolution rates of control and salt-stressed leaf pieces, a study was made of the effect of salt stress on two factors which influence oxygen evolution: stomatal aperture (Section 1.7.1), and chlorophyll content.

3.4.1 Stomata

The leaves of 7 day old control and salt-stressed barley were prepared for microscopic examination of stomata by two methods: epidermal strips and Xantopren impressions (Sections 2.8.1 and 2.8.2).

Epidermal strips and Xantopren impressions were taken from control and salt-stressed leaves which were growing in the growth room. The strips and impressions were taken from the same area of the whole leaf as was used, when required, for detached leaf pieces.

In addition, epidermal strips and Xantopren impressions were taken from control and salt-stressed leaf pieces which had been exposed to the atmosphere inside the chamber of the Leaf Disc Electrode for 10 minutes.

Table II shows the length and width of the stomatal pores (measured using epidermal strips only) of 7 day old control and salt-stressed barley leaves after exposure to the atmosphere inside the growth room and leaf chamber.

T A B L E II

The Length and Width of the Stomatal Pores of 7 Day Old Control and Salt-Stressed Barley, Measured Using Epidermal Strips

Measurements of the stomatal pores were carried out after exposing control and salt-stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using epidermal strips. The results indicate the mean \pm S.E.M. for 30 determinations.

[NaCl]mM	Length or Width of Stomatal Pore (μ m)	
	Growth Room	Leaf Chamber
Control	Length	23 \pm 0.8
	Width	2.8 \pm 0.21
250	Length	25 \pm 0.6
	Width	1.1 \pm 0.28
400	Length	28 \pm 0.6
	Width	1.2 \pm 0.20
600	Length	29 \pm 0.8
	Width	1.0 \pm 0.13
		28 \pm 0.5
		1.1 \pm 0.16

In Table III, the same measurements as in Table II were carried out, but in this case only Xantopren impressions were used.

The stomatal pores of control leaves from the growth room were shorter and wider (more open) than those of salt-stressed leaves from the growth room. In contrast, the stomatal pores of control leaf pieces from the leaf chamber were almost closed, as were those of salt-stressed leaf pieces from the leaf chamber.

Epidermal strips and Xantopren impressions gave similar results for the measurement of the stomatal pores of 7 day old control and salt-stressed barley. Since it sometimes proved difficult to remove epidermal strips from the barley leaves without damaging the strips themselves, only Xantopren impressions were used to measure the stomatal pores of 8 and 9 day old control and salt-stressed barley. These results are shown in Tables IV and V respectively.

Tables IV and V showed similar results to those of Table III. Therefore, more prolonged exposure of the leaves to salt did not seem to have a more pronounced effect on the shape of the stomata.

T A B L E III

The Length and Width of the Stomatal Pores of 7 Day Old Control and Salt-Stressed Barley, Measured Using Xantopren Impressions

Measurements of the stomatal pores were carried out after exposing control and salt-stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using Xantopren impressions. The results indicate the mean \pm S.E.M. for 30 determinations.

[NaCl]mM	Length or Width of Stomatal Pore (μ m)	
	Growth Room	Leaf Chamber
Control	Length	21 \pm 0.7
	Width	0.9 \pm 0.20
250	Length	27 \pm 0.4
	Width	0.5 \pm 0.16
400	Length	25 \pm 0.5
	Width	1.1 \pm 0.20
600	Length	28 \pm 0.7
	Width	0.6 \pm 0.17

T A B L E IV

The Length and Width of the Stomatal Pores of 8 Day Old Control and Salt-Stressed Barley, Measured Using Xantopren Impressions

Measurements of the stomatal pores were carried out after exposing control and salt-stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using Xantopren impressions. The results indicate the mean \pm S.E.M. for 30 determinations.

[NaCl]mM	Length or Width of Stomatal Pore (μ m)	
	Growth Room	Leaf Chamber
Control	Length	20 ± 0.4
	Width	2.2 ± 0.27
250	Length	25 ± 0.5
	Width	0.6 ± 0.18
400	Length	22 ± 1.4
	Width	27 ± 0.6
600	Length	22 ± 0.7
	Width	28 ± 0.7
600	Length	25 ± 0.7
	Width	27 ± 0.9
600	Length	27 ± 0.9
	Width	0.8 ± 0.15
600	Length	25 ± 0.7
	Width	27 ± 0.9
600	Length	27 ± 0.9
	Width	0.8 ± 0.15
600	Length	25 ± 0.7
	Width	27 ± 0.9

T A B L E V

The Length and Width of the Stomatal Pores of 9 Day Old Control and Salt-Stressed Barley, Measured Using Xantopren Impressions

Measurements of the stomatal pores were carried out after exposing control and salt-stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using Xantopren impressions. The results indicate the mean \pm S.E.M. for 30 determinations.

[NaCl]mM	Length or Width of Stomatal Pore (μ m)	
	Growth Room	Leaf Chamber
Control	Length	20 ± 0.6
	Width	27 ± 0.6
250	Length	2.2 ± 0.16
	Width	1.0 ± 0.16
400	Length	23 ± 0.7
	Width	27 ± 0.7
600	Length	24 ± 0.6
	Width	25 ± 1.0
600	Length	1.0 ± 0.14
	Width	0.9 ± 0.20
600	Length	27 ± 0.7
	Width	26 ± 0.9
600	Length	27 ± 0.7
	Width	26 ± 0.9
600	Length	27 ± 0.7
	Width	26 ± 0.9

T A B L E V

The Length and Width of the Stomatal Pores of 9 Day Old Control and Salt-Stressed Barley, Measured Using Xantopren Impressions

Measurements of the stomatal pores were carried out after exposing control and salt-stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using Xantopren impressions. The results indicate the mean \pm S.E.M. for 30 determinations.

[NaCl]mM	Length or Width of Stomatal Pore (μ m)	
	Growth Room	Leaf Chamber
Control	Length	20 ± 0.6
	Width	27 ± 0.6
250	Length	2.2 ± 0.16
	Width	1.0 ± 0.16
400	Length	23 ± 0.7
	Width	27 ± 0.7
600	Length	24 ± 0.6
	Width	25 ± 1.0
600	Length	1.0 ± 0.14
	Width	0.9 ± 0.20
600	Length	27 ± 0.7
	Width	26 ± 0.9
600	Length	27 ± 0.7
	Width	26 ± 0.9
600	Length	27 ± 0.7
	Width	26 ± 0.9

Meidner and Mansfield (1968) have derived a formula which can be used to estimate the rate of carbon dioxide intake into a leaf:

$$\begin{aligned} \text{Rate of CO}_2 \text{ intake} & \quad \Delta p \times C \times A \\ (\text{mg CO}_2) \cdot \text{cm}^{-2} \cdot \text{s}^{-1} & = \frac{\Delta p \times C \times A}{L + \frac{\pi D^{0.6}}{8} + \frac{A}{na} \left(1 + \frac{\pi d}{4} \right)} \end{aligned}$$

where,

Δp = CO₂ density in the growth room or leaf chamber

C = Diffusion coefficient for CO₂

A = Area of the whole leaf surface

L = Length of the diffusion path in the air space system of the leaf

D = Diameter of the whole leaf area

n = Number of stomata in the leaf surface

a = Cross-sectional area of one stoma

l = Length or depth of the stomatal tube

d = Diameter of the stomatal tube

Using the mean values obtained in Tables III, IV and V for the length and width of the stomatal pores, the calculated rate of carbon dioxide intake by each set of control and salt-stressed barley was estimated from the formula above. Results are shown in Table VI.

T A B L E VI

Rates of Carbon Dioxide Intake by 7, 8 and 9 Day Old Control and Salt-Stressed Barley

Rates of carbon dioxide intake were calculated using the mean values for stomatal dimensions obtained in Tables III, IV and V. Results are given for leaves from the growth room (G.R.) and for leaf pieces from the leaf chamber (L.C.).

Rate of Carbon Dioxide Intake (mg CO ₂).cm ⁻² .s ⁻¹				
[NaCl]mM		7 Days Old	8 Days Old	9 Days Old
Control	G.R.	6.7 x 10 ⁻⁵	5.8 x 10 ⁻⁵	5.6 x 10 ⁻⁵
	L.C.	8.1 x 10 ⁻³	5.7 x 10 ⁻³	7.3 x 10 ⁻³
250	G.R.	5.0 x 10 ⁻⁵	5.3 x 10 ⁻⁵	4.7 x 10 ⁻⁵
	L.C.	5.2 x 10 ⁻³	6.0 x 10 ⁻³	7.1 x 10 ⁻³
400	G.R.	4.1 x 10 ⁻⁵	4.5 x 10 ⁻⁵	4.0 x 10 ⁻⁵
	L.C.	8.9 x 10 ⁻³	7.8 x 10 ⁻³	6.4 x 10 ⁻³
600	G.R.	4.9 x 10 ⁻⁵	4.7 x 10 ⁻⁵	3.7 x 10 ⁻⁵
	L.C.	7.0 x 10 ⁻³	8.7 x 10 ⁻³	8.5 x 10 ⁻³

When control and salt-stressed leaves from the growth room were compared, there appeared to be a general trend; the higher the level of salt stress, the lower the calculated rate of carbon dioxide intake. There was no consistent difference in the rates of carbon dioxide intake between control and salt-stressed leaf pieces from the chamber, but the actual rate of each set of leaf pieces from the chamber was 100 times faster than that of any leaf set from the growth room. Increased exposure of the leaves to salt did not have a more pronounced effect on the rates of carbon dioxide intake.

3.4.2 Chlorophyll Content

The chlorophyll contents of salt-stressed barley leaf pieces were compared with those of control leaf pieces (Figure 28).

Both control and salt-stressed leaf pieces showed an increase in chlorophyll content with age. In each of the three sets of leaves (7, 8 and 9 day old), increasing the external [NaCl] caused an appreciable decline in the chlorophyll content.

3.4.3 Oxygen Evolution

The steady-state rates of oxygen evolution by control leaf pieces were compared with those of salt-stressed leaf pieces (Table VII).

As the external [NaCl] increased, the rate of oxygen evolution showed a marked decrease. More prolonged exposure of the leaves to salt did not have a more pronounced effect on the rate of oxygen evolution.

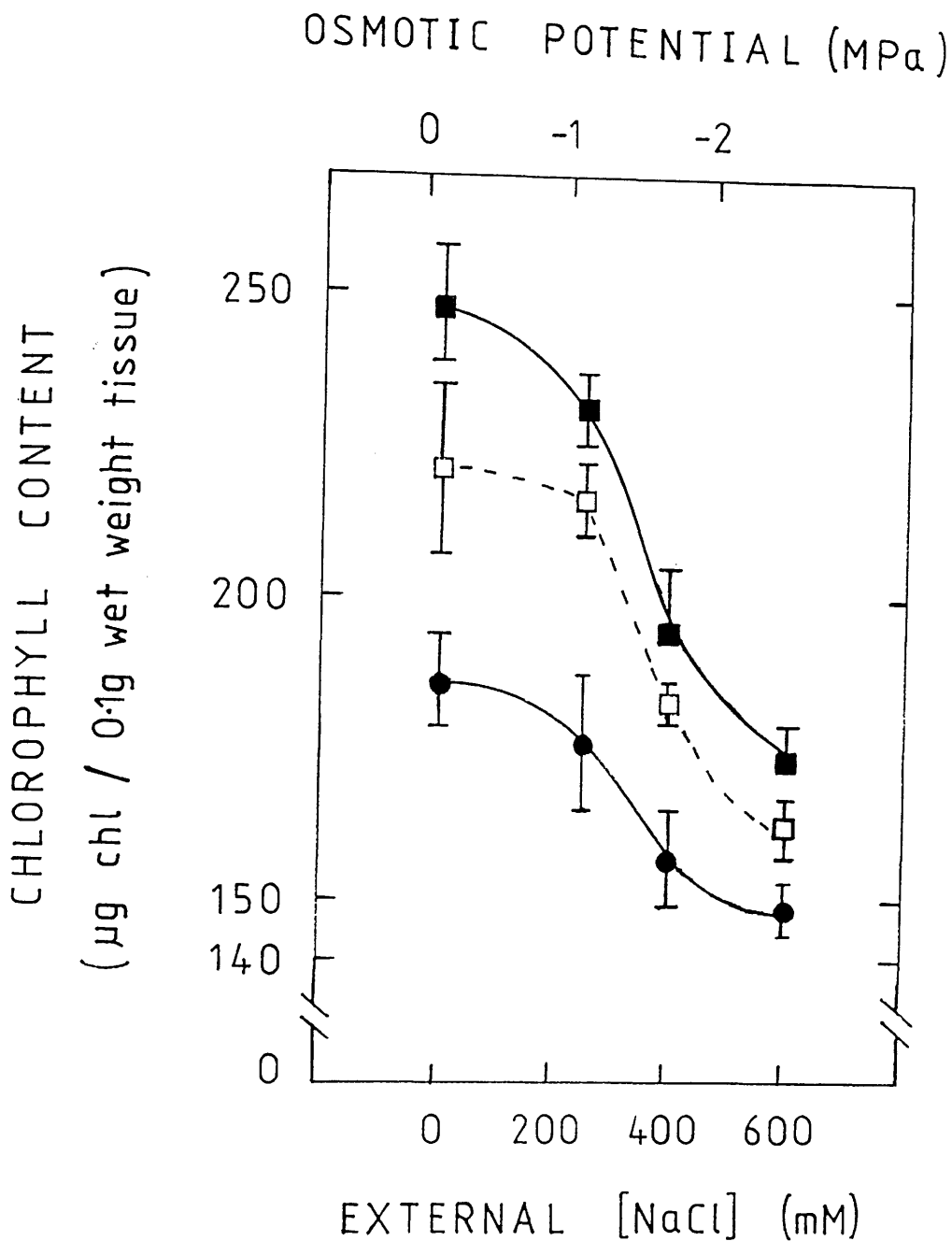


Figure 28. The effect of increasing the external [NaCl] on the chlorophyll content of 7 (●—●), 8 (□—□) and 9 (■—■) day old barley leaf pieces. Each point represents the mean \pm S.E.M. of 3 determinations.

T A B L E VII

The Effect of Increasing the External [NaCl] on the Steady-State Rates of Oxygen Evolution by 7, 8 and 9 Day Old Barley Leaf Pieces

Leaf pieces were illuminated with broad-band blue light (Corning 4.76, Corning 4.96 and Balzers Calflex C filters) of intensity 158Wm^{-2} . The results indicate the mean \pm S.E.M. for 4 determinations.

Oxygen Evolution Rate ($\mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{h}^{-1}$)			
[NaCl]mM	7 Days Old	8 Days Old	9 Days Old
Control	222 \pm 15.3	224 \pm 11.7	216 \pm 10.6
250	184 \pm 11.8	193 \pm 9.9	177 \pm 8.1
400	158 \pm 14.5	172 \pm 7.6	121 \pm 9.2
600	118 \pm 9.5	113 \pm 12.8	119 \pm 12.4

3.4.4 Photoinhibition

To check that the lower oxygen evolution rates of the salt-stressed leaf pieces were not simply the result of these leaf pieces being more liable to photoinhibition than the control leaf pieces, oxygen evolution rates of 8 day old control and 600mM NaCl-stressed leaf pieces were monitored over a period (> 2 hours) of continuous illumination (Figure 29).

Control and salt-stressed leaf pieces showed a similar pattern of oxygen evolution over a long period of time. During the first 2 or 3 minutes of illumination, the rate increased slightly and then remained constant until 50 or 60 minutes after illumination. The rate then fell sharply and continued to fall more gradually until the end of the experimental period.

3.4.5 Discussion

In the atmosphere of the growth room (0.03% CO_2), the stomatal pores of the salt-stressed leaves were less open than those of the control leaves. This would probably explain the lower rates of carbon dioxide intake by the salt-stressed leaves.

In the atmosphere of the chamber of the Leaf Disc Electrode (5% CO_2), the stomatal pores of both control and salt-stressed leaf pieces were almost closed but the rates of carbon dioxide intake by these leaf pieces were still 100 times faster than the rates shown by the leaves in the growth room. Presumably, the very large carbon dioxide diffusion gradient in the chamber meant that the closed stomata offered only minimal resistance to the fast intake of carbon dioxide. In consequence, the lower rates of oxygen evolution by the salt-stressed leaf

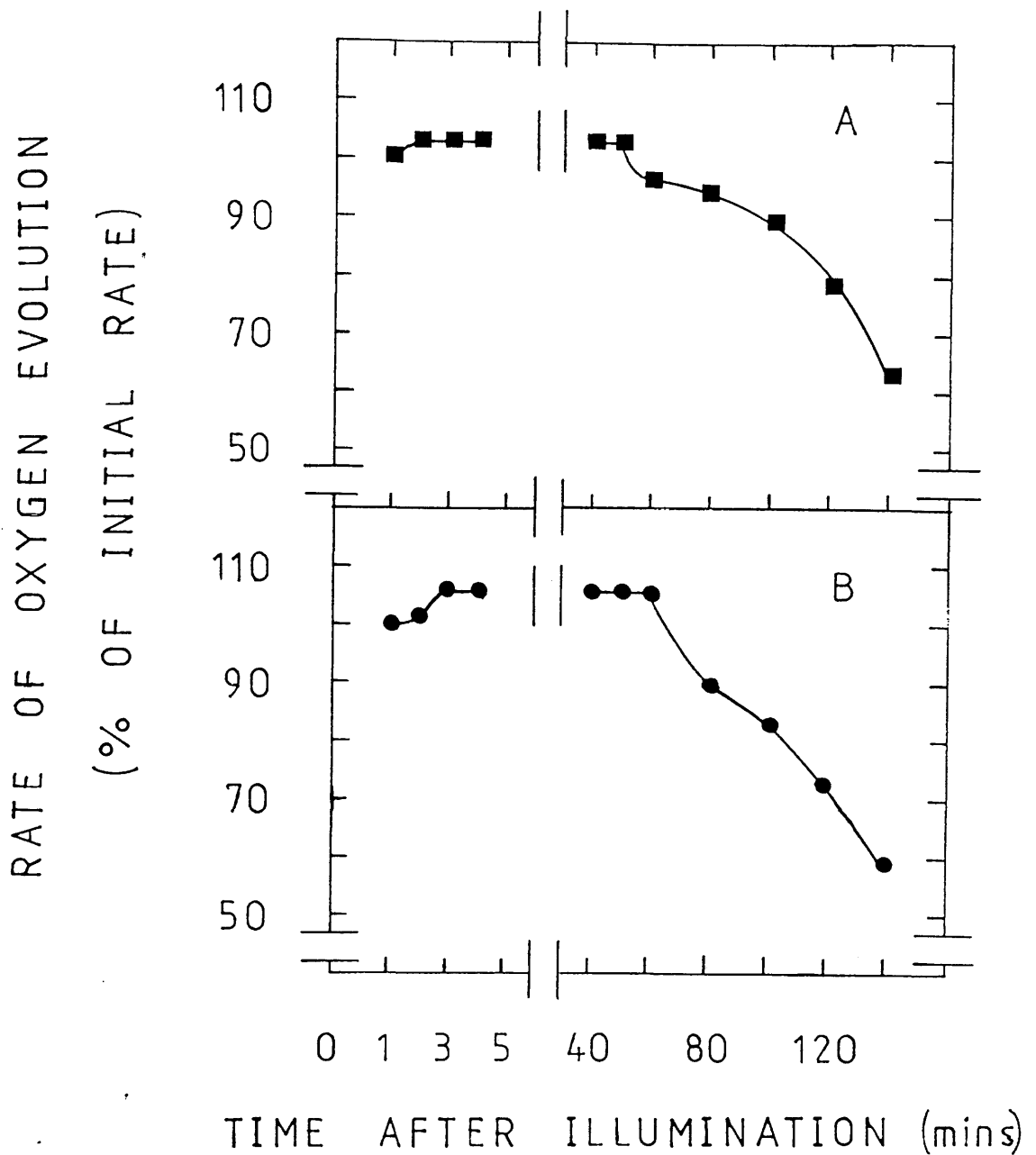


Figure 29. Oxygen evolution rates of 8 day old control and salt-stressed barley leaf pieces during an illumination period of 140 minutes. A) Control: initial rate = $187 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{h}^{-1}$; B) 600mM NaCl-stressed leaves: initial rate = $113 \mu\text{moles O}_2 \cdot (\text{mg chl})^{-1} \cdot \text{h}^{-1}$. Results indicate the mean \pm S.E.M. of 3 determinations. Standard error bars were within the symbol size.

pieces could not simply be due to salt having caused an inhibition in carbon dioxide intake by inducing stomatal closure.

Similarly, the lower rates of oxygen evolution by the salt-stressed leaf pieces could not be due to photoinhibition since control and salt-stressed leaf pieces showed similar patterns of oxygen evolution over a long period of time. The sharp fall in the rate of oxygen evolution after one hour may signify that the carbon dioxide supply in the leaf chamber was becoming depleted.

These results suggest that salt inhibits oxygen evolution by a mechanism other than stomatal closure or photoinhibition.

3.5 The Effect of Increasing the External [NaCl] on the Chlorophyll Fluorescence Induction Kinetics of Barley Leaf Pieces

When the Kautsky chlorophyll fluorescence transients (Section 1.8.1) of control and salt-stressed barley leaf pieces were compared, the most obvious difference was the height of the S-M transient relative to the height of the P peak. This was calculated as is shown below.

For the chlorophyll fluorescence transient shown in Figure 30:

Height of the S-M transient

relative to the height of the P peak = $\frac{a}{b} \times 100$

(relative units)

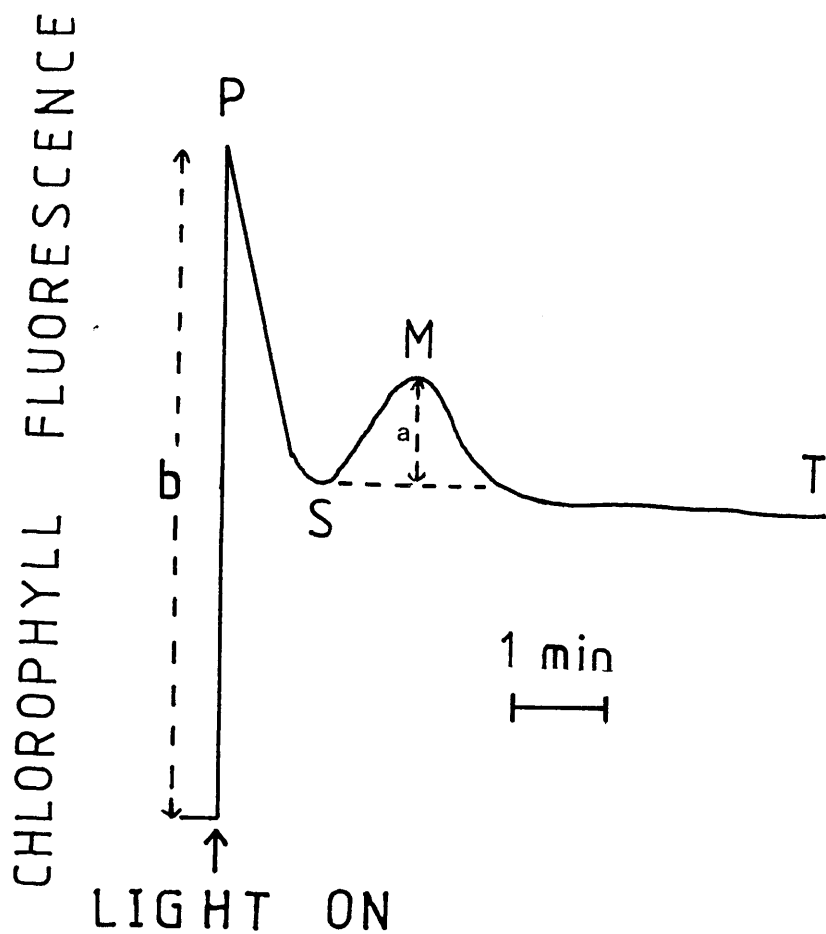


Figure 30. Chlorophyll fluorescence induction curve for control barley leaf pieces following a dark period of 10 minutes. The leaf pieces were illuminated with broad-band blue light (Corning 4.76, Corning 4.96 and Balzers Calflex C filters) of intensity 158Wm^{-2} . Chlorophyll fluorescence was detected by a photodiode protected by a Wratten 88A filter. (a) represents the height of the S-M transient and (b) represents the height of P.

Results for 7, 8 and 9 day old control and salt-stressed barley leaf pieces are shown in Table VIII. There appeared to be a general trend; the higher the external [NaCl], the lower the height of the S-M transient. Increased exposure of the leaf pieces to salt did not have a more pronounced effect on the height of the S-M transient.

3.6 The Effect of Increasing the External [NaCl] on the Redox State of Q During Kautsky Transients in Barley Leaf Pieces: "Light Doubling"

The results of Sections 3.4.3 and 3.5 suggest that NaCl has a marked effect on oxygen evolution and on the height of the S-M chlorophyll fluorescence transient. While it may be interesting to study the changes induced by NaCl stress, it is perhaps more important to try to determine exactly what underlying photosynthetic mechanisms NaCl is affecting to bring about these changes. One underlying factor in the chloroplast which NaCl may affect is the redox state of Q. The yield of chlorophyll fluorescence during a Kautsky oscillation can be used to measure the redox state of Q because quenching is governed by a number of factors, including the proportion of oxidised Q (q_Q). In whole leaves, q_Q can be measured during a chlorophyll fluorescence transient by applying a second light of high intensity at appropriate intervals and observing the resultant fast chlorophyll fluorescence induction kinetics (Bradbury and Baker, 1981). This technique is known as "light doubling". Quick and Horton (1984b) have used a modification of this light doubling technique to monitor the changes in q_Q that occur during the Kautsky oscillation in barley protoplasts. A similar approach to that of Quick and Horton (1984b) has been used

T A B L E VIII

The Effect of Increasing the External [NaCl] on the Height of the S-M Chlorophyll Fluorescence Transient Relative to the Height of the P Peak

Results were calculated as described in the text for Figure 30 and indicate the mean \pm S.E.M. for 9 determinations.

Height of S-M Transient Relative to Height of P
(Relative Units)

[NaCl]mM	7 Days Old	8 Days Old	9 Days Old
Control	10 \pm 1.0	12 \pm 1.7	9 \pm 1.6
250	8 \pm 1.7	12 \pm 2.5	7 \pm 1.8
400	5 \pm 1.0	7 \pm 1.6	5 \pm 0.7
600	2 \pm 0.5	3 \pm 0.7	6 \pm 1.4

here:

- a) to study the changes in the redox state of Q during the chlorophyll fluorescence induction curve of barley control leaf pieces by calculating q_Q at frequent intervals during the induction curve.
- b) to study the effect of NaCl on the redox state of Q during the chlorophyll fluorescence induction curve of barley leaf pieces.

Barley leaf pieces were dark-adapted for 10 minutes and then illuminated with modulated blue-green light to induce Kautsky transients. During the course of the transients, non-modulated blue-green light was applied to the system in the form of 1 second light pulses every 10 seconds (Section 2.14.1).

In these light doubling experiments, the system used to detect chlorophyll fluorescence consisted of a photodiode connected to a lock-in amplifier. The lock-in amplifier was tuned to the frequency of the modulated excitation light (189 Hz) and monitored only chlorophyll fluorescence emitted at this frequency i.e. only modulated chlorophyll fluorescence. The change in the intensity of the excitation light as a result of applying the non-modulated light pulses was not monitored by the detection system since the light pulses produced only non-modulated chlorophyll fluorescence. Thus any fluctuation in the modulated chlorophyll fluorescence signal as a result of applying the light pulses was due to the light pulses having altered only the yield of the modulated chlorophyll fluorescence i.e. only the redox state of Q.

The light pulses were found to induce a rapid increase in the chlorophyll fluorescence signal (Figure 31). This rapid increase was due to photoreduction of the fraction of Q that was in the oxidised state before the light pulses were applied.

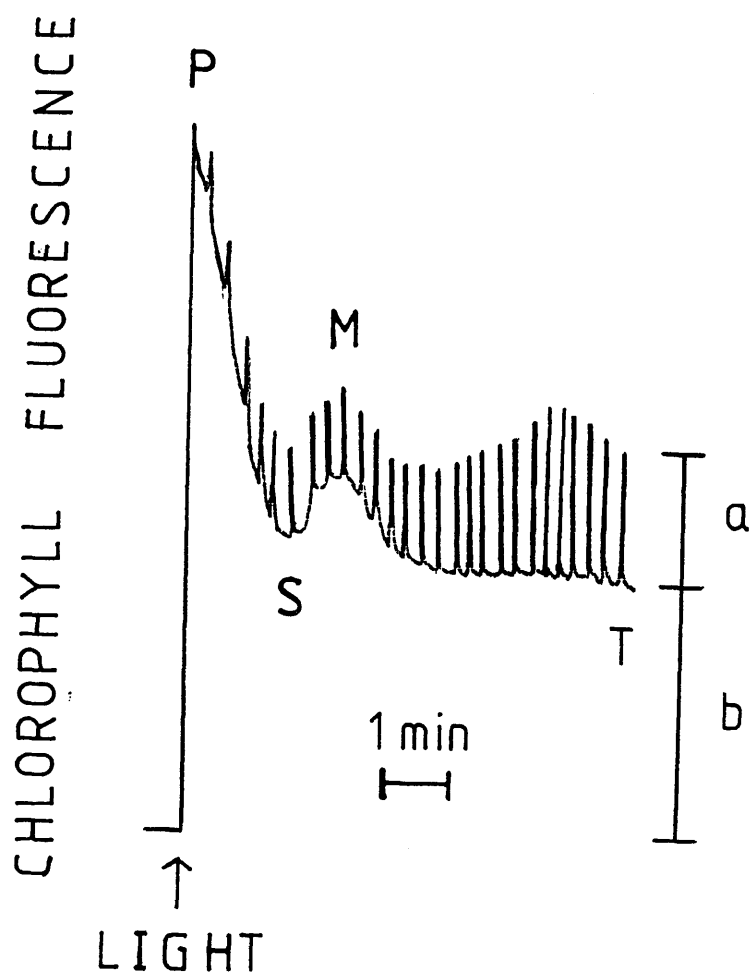


Figure 31. The effect of applying 1 second light pulses every 10 seconds to the chlorophyll fluorescence induction curve of control barley leaf pieces following a 10 minute dark period. The induction curve was initiated by illuminating the leaf pieces with modulated blue-green light (via Corning 4.76 and 4.96 filters) of intensity 21Wm^{-2} . The modulated chlorophyll fluorescence signal, transmitted by a Wratten 88A filter, was detected by a photodiode coupled to a lock-in amplifier operating at 189Hz. When required, the non-modulated blue-green light pulses (filters as above) were introduced at an intensity of 12.5Wm^{-2} .

Calculation of q_Q was achieved by measuring the ratio of the size of the pulse height to the chlorophyll fluorescence level immediately before the pulse. These values were then converted into percentages. For example, the calculation of the % q_Q at T in Figure 31 was carried out as below:

$$\begin{aligned}\% q_Q &= \frac{a}{b} \times 100 \\ &= \frac{17 \text{ units}}{34 \text{ units}} \times 100 \\ &= 50\end{aligned}$$

Dividing the pulse height by the chlorophyll fluorescence level immediately before the pulse gave a measure of the relative increase in the yield of chlorophyll fluorescence induced by the light pulses, not the absolute increase. Measurement of relative rather than absolute values enabled the comparison of the % q_Q from different samples.

To make this a useful method of determining q_Q , the intensity of the pulses must be sufficient to close a larger proportion, if not all, of the PSII centres (Quick and Horton, 1984b). This was studied by varying the intensity of the light pulses at steady-state T and studying the effect on q_Q (Figure 32). Saturation of q_Q was achieved at a light intensity of 11.6 W m^{-2} (light intensity used in the experiments was 12.5 W m^{-2}).

Table IX shows the % q_Q at 10 second intervals along the chlorophyll fluorescence transients of 7 day old control and salt-stressed barley leaves and, in Figure 33, the same results are presented graphically for control and 600mM salt-stressed leaves. The time taken to reach the S chlorophyll fluorescence level and

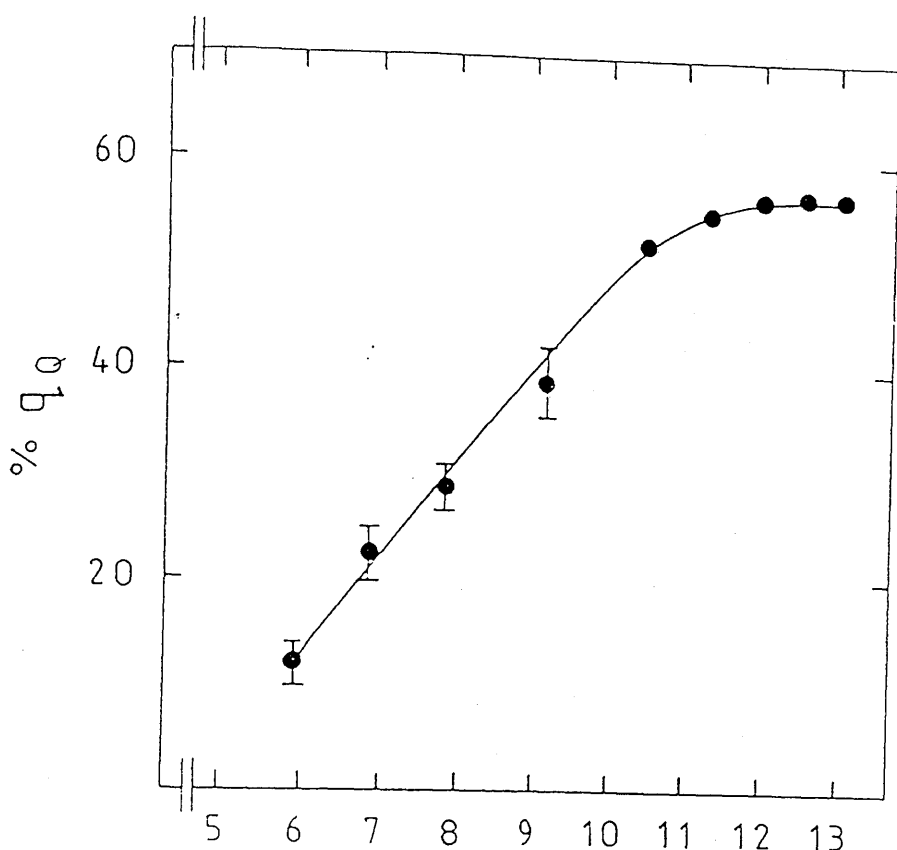


Figure 32. The effect of increasing the intensity of the light pulses on the % q_Q of control barley leaf pieces. Chlorophyll fluorescence induction was initiated after a 10 minute dark period by illuminating the leaf pieces with modulated blue-green light (via Corning 4.76 and 4.96 filters) of intensity 21Wm^{-2} . The modulated chlorophyll fluorescence signal, transmitted by a Wratten 88A filter, was detected by a photodiode coupled to a lock-in amplifier operating at 189Hz. After 6 minutes, at T, the non-modulated blue-green light pulses (filters as above) were introduced and their intensity was adjusted by means of neutral density filters. Each point represents the mean \pm S.E.M. of 3 determinations. Where standard error bars are not shown, errors were within the symbol size.

T A B L E IX

Measurement of % q_Q at 10 Second Intervals Along the Chlorophyll Fluorescence Induction Curve of 7 Day Old Control and Salt-Stressed Barley Leaves

S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. for 9 determinations.

Seconds After Illum.	% q_Q			
	Control	250mM NaCl	400mM NaCl	600mM NaCl
10	19 \pm 1.7	14 \pm 2.4	8 \pm 1.2	7 \pm 0.6
20	23 \pm 1.0	19 \pm 3.4	10 \pm 1.8	8 \pm 0.9
30	28 \pm 1.1	26 \pm 5.1	14 \pm 2.8	10 \pm 1.5
40	33 \pm 1.5	30 \pm 5.0	19 \pm 3.7	13 \pm 2.4
50	38 \pm 3.3	35 \pm 5.2	25 \pm 5.0	16 \pm 3.3
60	S 42 \pm 3.4	38 \pm 5.1	31 \pm 6.0	21 \pm 3.9
70	45 \pm 4.4	S 38 \pm 4.1	37 \pm 6.1	25 \pm 4.6
80	47 \pm 4.7	38 \pm 3.2	S 40 \pm 6.1	28 \pm 4.7
90	49 \pm 5.0	37 \pm 2.9	38 \pm 4.7	32 \pm 4.5
100	40 \pm 3.8	35 \pm 2.8	36 \pm 3.8	S 35 \pm 4.3
110	35 \pm 3.5	34 \pm 3.3	34 \pm 3.5	36 \pm 3.6
120	33 \pm 2.8	33 \pm 3.5	34 \pm 3.5	34 \pm 3.3
130	M 32 \pm 2.6	M 35 \pm 4.1	34 \pm 4.0	33 \pm 2.9
140	34 \pm 2.2	37 \pm 4.3	M 36 \pm 4.1	34 \pm 3.7
150	37 \pm 2.4	39 \pm 5.2	38 \pm 4.2	33 \pm 3.6
160	40 \pm 2.7	41 \pm 5.1	40 \pm 4.8	M 32 \pm 3.7
170	47 \pm 3.4	41 \pm 5.2	41 \pm 4.7	35 \pm 3.7
180	48 \pm 3.5	44 \pm 3.6	43 \pm 4.2	39 \pm 3.8
190	47 \pm 3.6	46 \pm 5.1	44 \pm 4.1	38 \pm 4.2
360	T 56 \pm 6.2	T 55 \pm 5.9	T 50 \pm 5.5	T 49 \pm 5.0

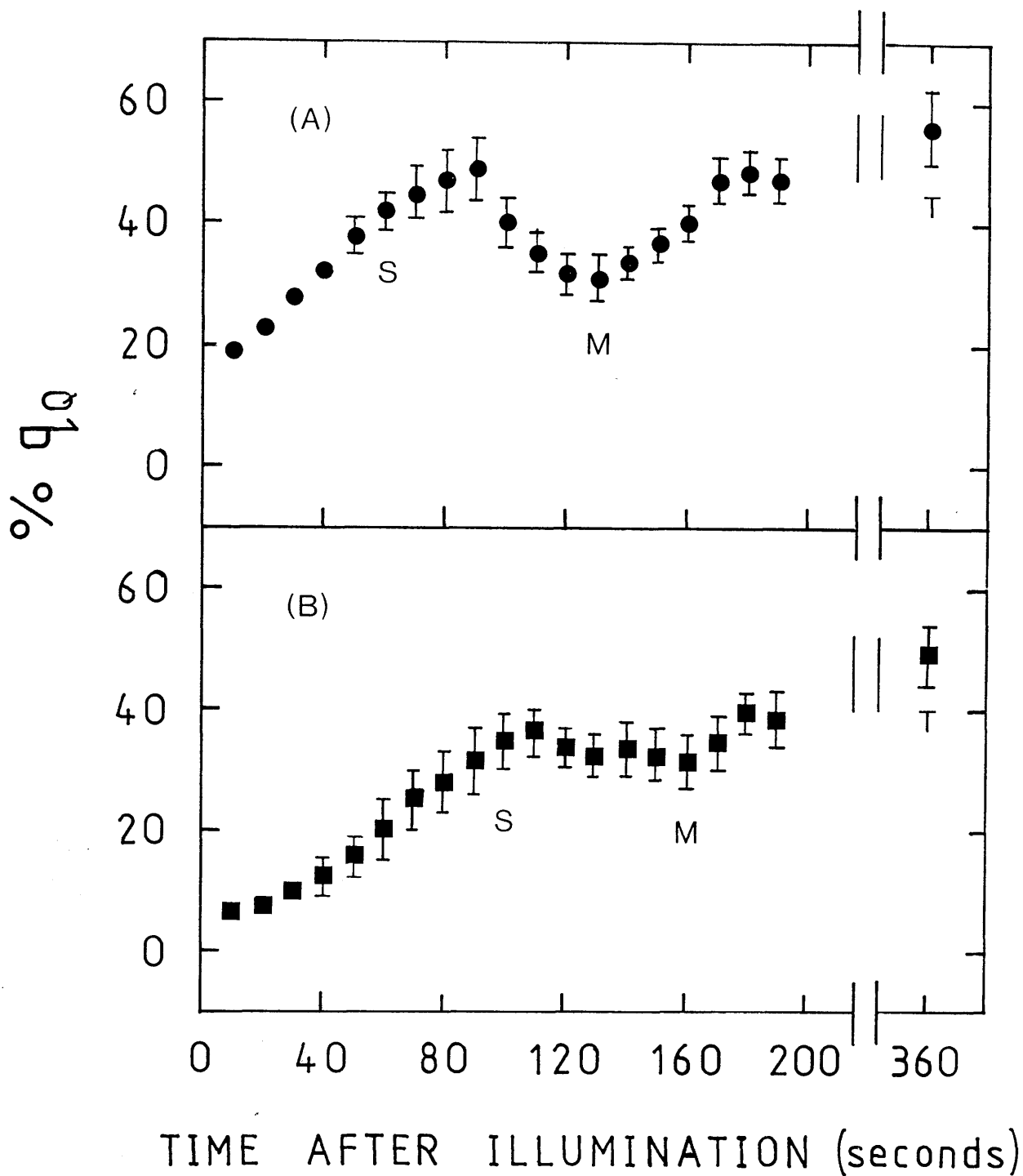


Figure 33. Measurement of % q_Q at 10 second intervals along the chlorophyll fluorescence induction curve of 7 day old barley leaf pieces. A) Control; B) 600mM NaCl. S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. of 9 determinations. Where standard error bars are not shown, errors were within the symbol size.

the top of the M peak are also shown. The value of % q_Q at steady-state T was measured after 6 minutes. Table X and Figure 34, and Table XI and Figure 35 give the corresponding data for 8 and 9 day old barley leaves.

At each salt concentration, only a small percentage of Q was in the oxidised state 10 seconds after illumination. However, during the phase P to S the percentage of oxidised Q increased. It then fell as chlorophyll fluorescence rose to the top of the M peak, only to rise again as chlorophyll fluorescence fell from the M peak. The percentage of Q in the oxidised state was greatest at the steady-state T.

When the P to S phase of control and salt-stressed leaf pieces was compared it could be seen that the percentage of Q in the oxidised state at each time interval decreased very markedly as the salinity increased. There was also a delay in the time taken to reach the S chlorophyll fluorescence level at the higher salinities.

During the S to M and M to T phases, the percentage of oxidised Q in the salt-stressed leaf pieces was often lower than in the control leaf pieces but the decrease was less marked than in the P to S phase. A delay in the time taken to reach the top of the M peak was observed at the higher salinities.

Table XII shows the difference in the percentage of oxidised Q between S and the top of the M peak for 7, 8 and 9 day old control and salt-stressed barley leaf pieces. The major effect of NaCl between S and M was on the extent to which the redox state of Q changed. In control leaves, the large difference in percentage of oxidised Q indicated that the redox state of Q had changed markedly during S to M. However, at higher salinities, the small difference in percentage of oxidised Q between S and M indicated that the redox state of Q had changed only slightly.

T A B L E X

Measurement of % q_Q at 10 Second Intervals along the Chlorophyll
Fluorescence Induction Curve of 8 Day Old Control and Salt-Stressed
Barley Leaves

S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. for 9 determinations.

Seconds After Illum.	% q_Q			
	Control	250mM NaCl	400mM NaCl	600mM NaCl
10	18 \pm 1.3	17 \pm 2.2	10 \pm 2.0	9 \pm 1.2
20	25 \pm 2.5	24 \pm 3.1	12 \pm 2.4	11 \pm 1.7
30	32 \pm 2.7	25 \pm 2.9	15 \pm 3.4	14 \pm 1.8
40	36 \pm 2.8	37 \pm 4.4	20 \pm 4.3	18 \pm 2.0
50	S 36 \pm 2.7	41 \pm 3.8	27 \pm 5.5	22 \pm 2.4
60	39 \pm 3.0	S 43 \pm 3.7	39 \pm 6.8	26 \pm 2.3
70	35 \pm 2.1	38 \pm 2.1	S 41 \pm 7.0	S 34 \pm 1.9
80	32 \pm 1.9	38 \pm 3.6	41 \pm 7.0	33 \pm 2.0
90	28 \pm 1.8	37 \pm 4.1	37 \pm 5.9	35 \pm 2.0
100	26 \pm 1.5	34 \pm 2.6	32 \pm 4.6	35 \pm 1.5
110	M 26 \pm 1.7	M 33 \pm 2.2	29 \pm 4.1	34 \pm 1.7
120	30 \pm 2.5	34 \pm 1.5	29 \pm 4.4	M 31 \pm 2.3
130	31 \pm 2.4	33 \pm 2.0	30 \pm 5.0	35 \pm 2.1
140	34 \pm 2.3	39 \pm 2.9	M 34 \pm 5.6	35 \pm 1.8
150	36 \pm 2.3	41 \pm 3.2	38 \pm 5.8	37 \pm 1.7
160	39 \pm 2.1	42 \pm 3.1	40 \pm 6.5	38 \pm 1.8
170	41 \pm 2.0	43 \pm 3.2	42 \pm 6.6	38 \pm 1.9
180	42 \pm 3.1	43 \pm 3.3	43 \pm 5.0	39 \pm 2.0
190	44 \pm 3.2	44 \pm 3.1	43 \pm 5.1	40 \pm 2.1
360	T 51 \pm 1.9	T 49 \pm 6.4	T 48 \pm 2.4	T 44 \pm 3.5

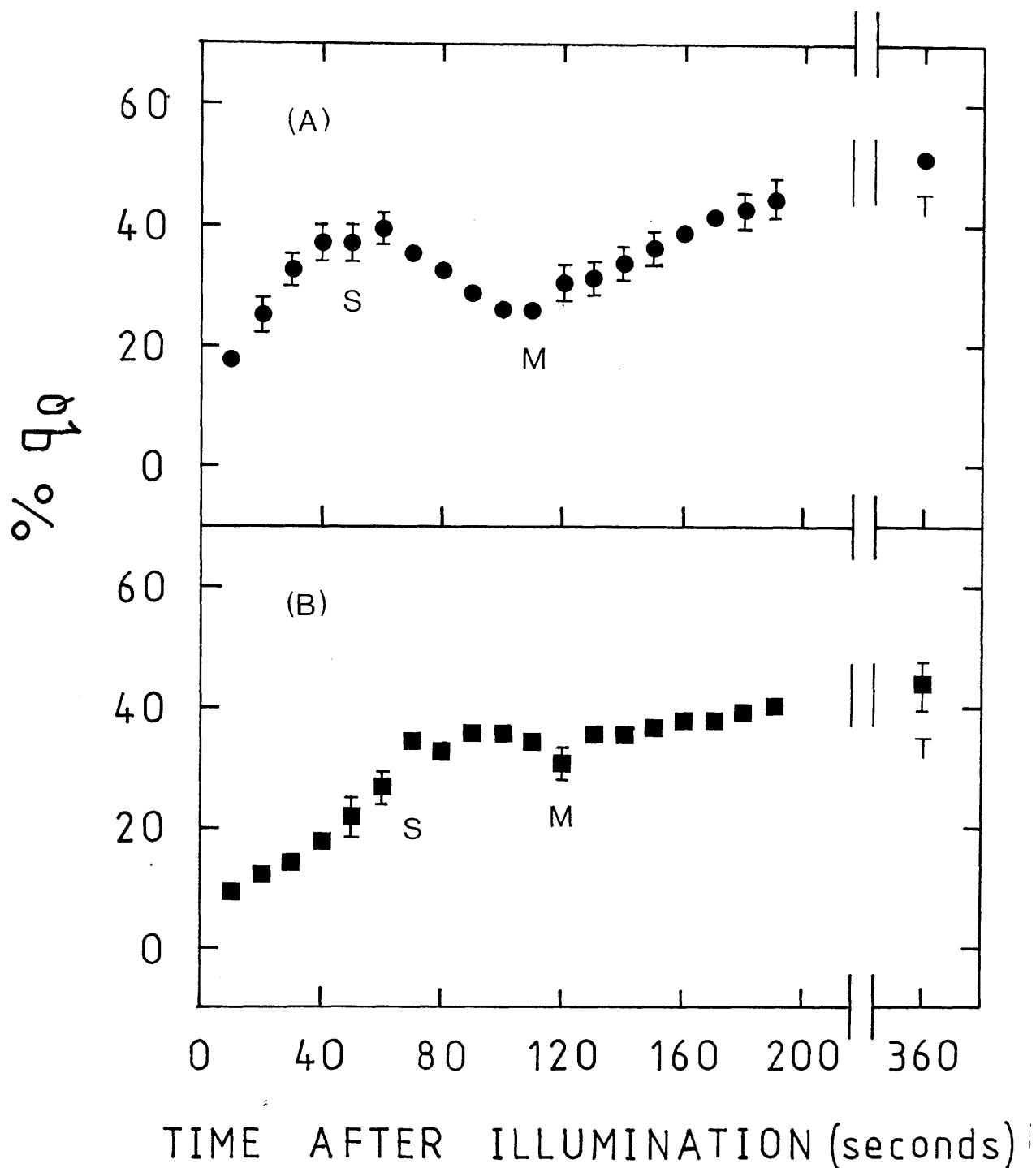


Figure 34. Measurement of % q_Q at 10 second intervals along the chlorophyll fluorescence induction curve of 8 day old barley leaf pieces. A) Control; B) 600mM NaCl. S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. of 9 determinations. Where standard error bars are not shown, errors were within the symbol size.

T A B L E X I

Measurement of % q_Q at 10 Second Intervals Along the Chlorophyll
Fluorescence Induction Curve of 9 Day Old Control and Salt-Stressed
Barley Leaves

S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. for 9 determinations.

Seconds After Illum.	% q_Q			
	Control	250mM NaCl	400mM NaCl	600mM NaCl
10	20 \pm 2.2	15 \pm 0.4	9 \pm 2.2	8 \pm 0.8
20	26 \pm 3.5	19 \pm 1.0	15 \pm 3.1	10 \pm 1.3
30	27 \pm 3.5	24 \pm 1.5	19 \pm 3.6	14 \pm 2.0
40	36 \pm 2.1	28 \pm 1.6	22 \pm 3.5	22 \pm 5.8
50	S 41 \pm 2.6	30 \pm 1.5	25 \pm 3.1	26 \pm 5.6
60	43 \pm 2.1	S 35 \pm 1.9	S 34 \pm 4.1	32 \pm 7.2
70	39 \pm 1.6	23 \pm 0.6	34 \pm 3.9	36 \pm 7.0
80	36 \pm 2.0	19 \pm 1.1	35 \pm 4.3	35 \pm 5.3
90	36 \pm 2.0	19 \pm 2.1	32 \pm 3.4	33 \pm 3.4
100	34 \pm 2.5	22 \pm 2.0	31 \pm 2.6	S 36 \pm 3.8
110	M 34 \pm 2.5	24 \pm 2.4	30 \pm 2.6	30 \pm 0.9
120	35 \pm 2.1	23 \pm 1.3	31 \pm 2.8	30 \pm 0.3
130	38 \pm 2.5	M 30 \pm 0.8	M 31 \pm 2.4	33 \pm 1.2
140	39 \pm 2.8	31 \pm 0.5	33 \pm 2.2	34 \pm 2.5
150	41 \pm 3.0	31 \pm 0.6	34 \pm 2.1	M 31 \pm 1.7
160	42 \pm 3.1	32 \pm 1.4	36 \pm 2.3	38 \pm 3.9
170	43 \pm 3.2	33 \pm 2.1	36 \pm 2.2	38 \pm 3.0
180	45 \pm 4.0	34 \pm 1.9	37 \pm 2.6	42 \pm 3.6
190	46 \pm 4.1	34 \pm 2.1	37 \pm 2.8	42 \pm 3.8
360	T 54 \pm 3.7	T 48 \pm 3.5	T 44 \pm 2.9	T 49 \pm 1.5

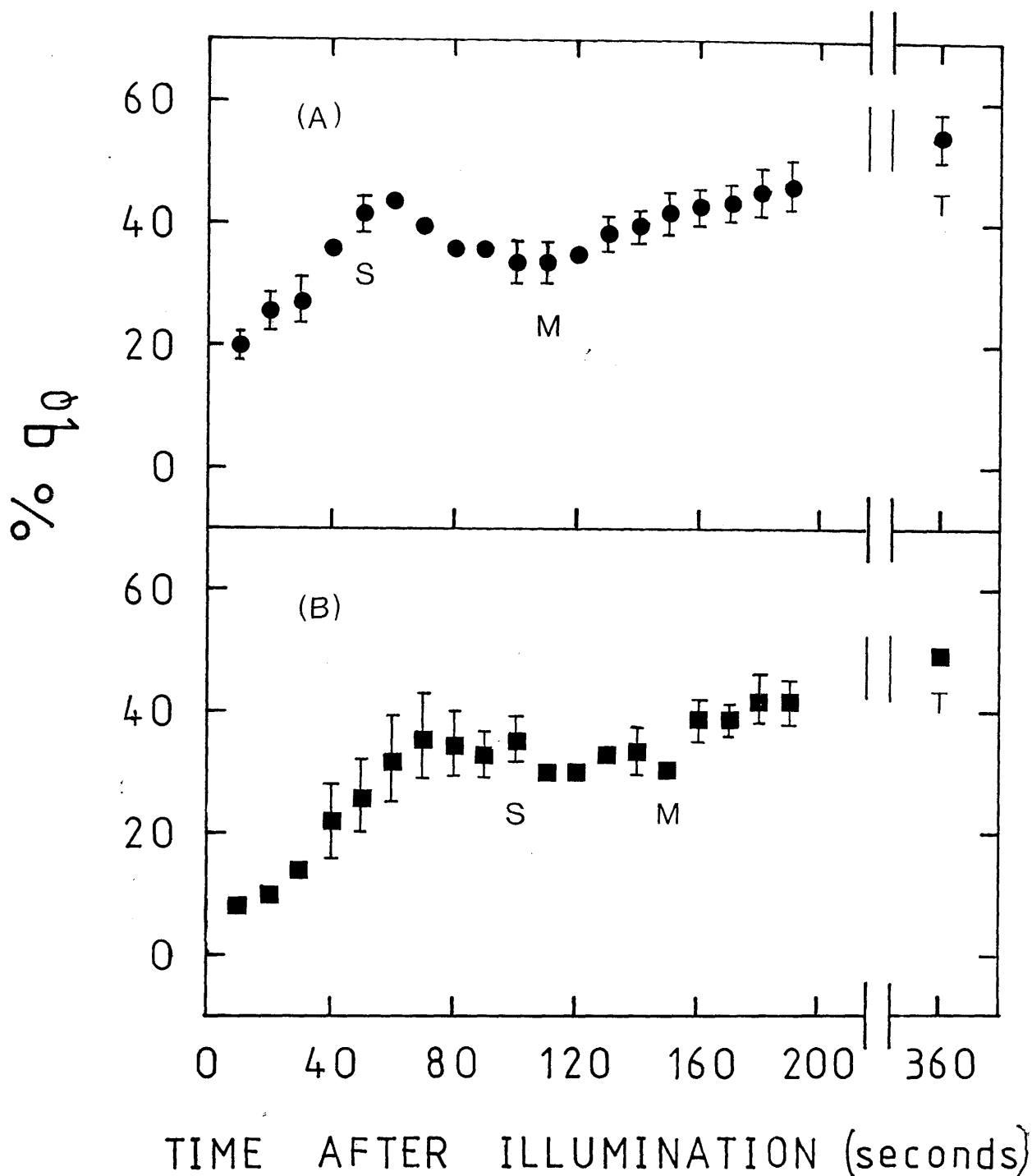


Figure 35. Measurement of % q_Q at 10 second intervals along the chlorophyll fluorescence induction curve of 9 day old barley leaf pieces. A) Control; B) 600mM NaCl. S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. of 9 determinations. Where standard error bars are not shown, errors were within the symbol size.

T A B L E XII

Difference in the percentage of oxidised Q between S and the top of the M peak for 7, 8 and 9 day old control and salt-stressed barley leaves. Results are in relative units and indicate the mean \pm S.E.M. for 9 determinations.

<u>[NaCl]</u> <u>mM</u>	Difference in the percentage of oxidised Q between S and the top of the M peak (relative units)		
	7 Days Old	8 Days Old	9 Days Old
0	10 \pm 3.2	10 \pm 2.5	7 \pm 3.0
250	3 \pm 2.4	10 \pm 5.5	5 \pm 1.8
400	4 \pm 4.1	7 \pm 2.8	3 \pm 3.3
600	3 \pm 1.7	3 \pm 3.4	5 \pm 4.1

Increased leaf age did not seem to have a pronounced effect on these light doubling results.

3.6.1 Discussion

The changes in the redox state of Q during the chlorophyll fluorescence induction curves of barley leaf pieces were very similar to those observed previously for barley protoplasts (Quick and Horton, 1984b). Immediately after P, Q was very reduced but during the chlorophyll fluorescence decrease from P to S, Q became very oxidised. As chlorophyll fluorescence rose to M, Q became re-reduced but was then re-oxidised as chlorophyll fluorescence fell to T. These results support the hypothesis of quenching during the induction period, which was described in Section 1.8.1.

The decreased percentage of Q in the oxidised state all through the chlorophyll fluorescence induction curves of salt-stressed barley, and particularly during P to S quenching, suggests that salt stress is having one of three effects; a stimulation in the activity of PSII, an inhibition in the activity of PSI or an inhibition of electron transport between Q and PSI. The decrease in the extent to which q_Q changed during the phase S to M at high salt concentrations reflects the decreased height of the M peak at high salt concentrations. A more reduced state of Q suggests that the decreased height of the M peak is the result of an increase in one of the quenching mechanisms other than q_Q , perhaps q_e (Section 1.8.1). An increase in q_e might be caused by an inhibition in one or more reactions of the RPP pathway resulting in less ATP being consumed. A delay in the RPP pathway starting up might

explain the delay in the time taken to reach the S and M chlorophyll fluorescence levels at high salt concentrations.

A stimulation in the activity of PSII could be due to an increase in the amount of light energy entering PSII. Similarly, an inhibition in PSI could be due to a decrease in the amount of light energy entering PSI. In the following experiments, therefore, the effect of NaCl on the distribution of light energy between PSI and PSII was studied.

3.7 The Effect of Increasing the External [NaCl] on State 1 - State 2 Transitions in Barley Leaf Pieces

State 1 - State 2 transitions (Section 1.5.5) in barley leaf pieces were studied by over-exciting each photosystem in turn; this induced the leaf pieces to alter the distribution of light energy between the two photosystems, and so maintain a balance in the excitation energy entering each photosystem. The effect of increasing the external [NaCl] on the efficiency of this redistribution process was then studied.

Barley leaf pieces were dark-adapted for 10 minutes and then illuminated with modulated Light 2 (which preferentially excited PSII) to induce Kautsky transients. After 10 minutes, non-modulated Light 1 (which preferentially excited PSI) was superimposed on Light 2. After a further 10 minutes Light 1 was turned off.

The system used to detect chlorophyll fluorescence was the same as that used for the light doubling experiments (Section 3.6) so that any fluctuation in the modulated chlorophyll fluorescence signal as a result of applying non-modulated Light 1 was due to Light 1 having altered only the yield of modulated

chlorophyll fluorescence. In addition, the use of modulated fluorimetry ensured that none of the red 715nm exciting light which may have passed through the 695nm interference filter in the photodiode was amplified.

Figure 36 shows a typical State 1 - State 2 chlorophyll fluorescence curve for control barley leaf pieces. Turning on Light 1 induced an immediate small decrease in the chlorophyll fluorescence level. After this initial decrease, the chlorophyll fluorescence level increased slowly and then levelled off at a slightly higher level than before. When Light 1 was turned off there was an immediate large rise in the chlorophyll fluorescence signal, followed by a rapid fall in chlorophyll fluorescence to the steady-state level.

Figures 37, 38 and 39 show typical State 1 - State 2 chlorophyll fluorescence curves for control and salt-stressed barley leaf pieces at 7, 8 and 9 days old respectively.

The major effects of NaCl on the State 1 - State 2 chlorophyll fluorescence curves appeared to be to cause a more gradual and smaller increase in chlorophyll fluorescence when Light 1 was turned on, a reduction in the height of the rapid chlorophyll fluorescence signal when Light 1 was turned off and a decrease in the rate of decline of the chlorophyll fluorescence signal to the steady-state level.

The curves of 7 and 8 day old leaf pieces were similar but 9 day old leaf pieces appeared to show more pronounced salt-induced changes. For instance, there was often no slow increase in the chlorophyll fluorescence signal at the highest salinities when Light 1 was turned on. In addition, when Light 1 was turned off, the immediate rise in chlorophyll fluorescence was often very small.

CHLOROPHYLL FLUORESCENCE

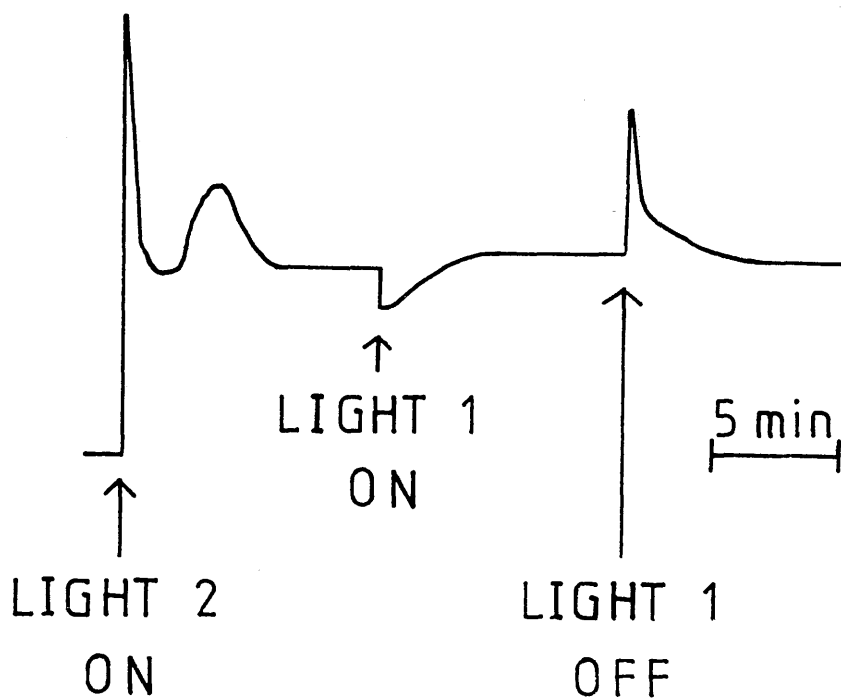


Figure 36. A typical State 1 - State 2 chlorophyll fluorescence curve for control barley leaf pieces following a 10 minute dark period. The induction curve was initiated by illuminating the leaf pieces with modulated blue-green light (Light 2), via Corning 4.76 and 4.96 filters, to give an intensity of 21Wm^{-2} . The modulated chlorophyll fluorescence signal, transmitted by a Balzers 695nm interference filter, was detected by a photodiode coupled to a lock-in amplifier operating at 189Hz. When required, non-modulated Light 1 (transmitted by a Schott RG 715 long-pass filter) was introduced at an intensity of 7Wm^{-2} .

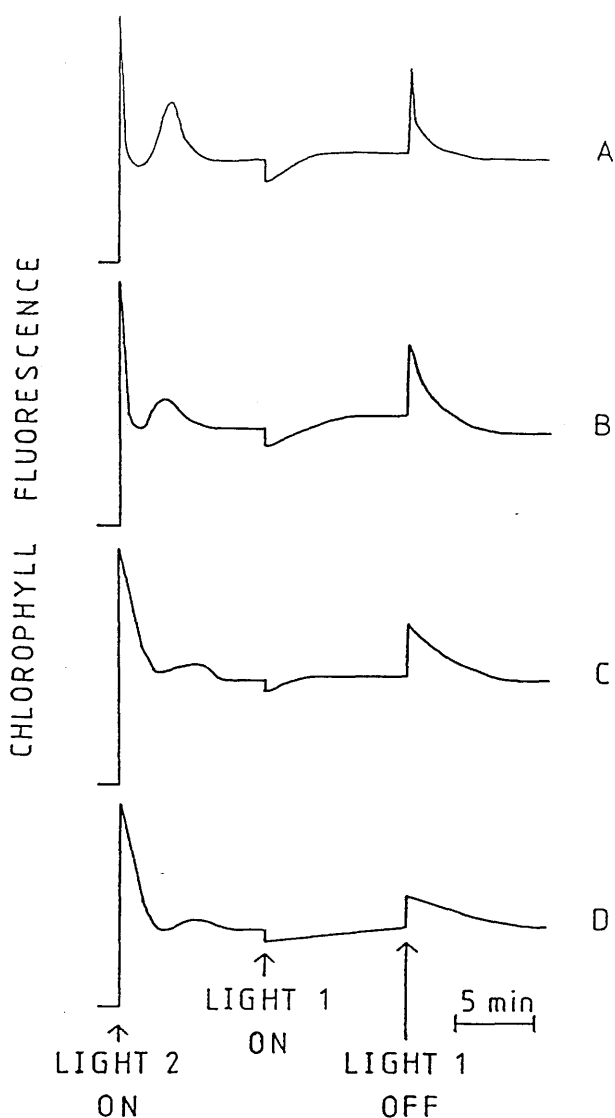


Figure 37. The effect of increasing the external [NaCl] on the State 1 - State 2 chlorophyll fluorescence transients of 7 day old barley leaf pieces. A) Control; B) 250mM NaCl; C) 400mM NaCl; D) 600mM NaCl. The induction curve was initiated by illuminating the leaf pieces with modulated blue-green light (Light 2), via Corning 4.76 and 4.96 filters, to give an intensity of 21Wm^{-2} . The modulated chlorophyll fluorescence signal, transmitted by a Balzers 695nm interference filter, was detected by a photodiode coupled to a lock-in amplifier operating at 189Hz. When required, non-modulated Light 1 (transmitted by a Schott RG 715 long-pass filter) was introduced at an intensity of 7Wm^{-2} . Each curve is representative of an experiment which was replicated at least five times.

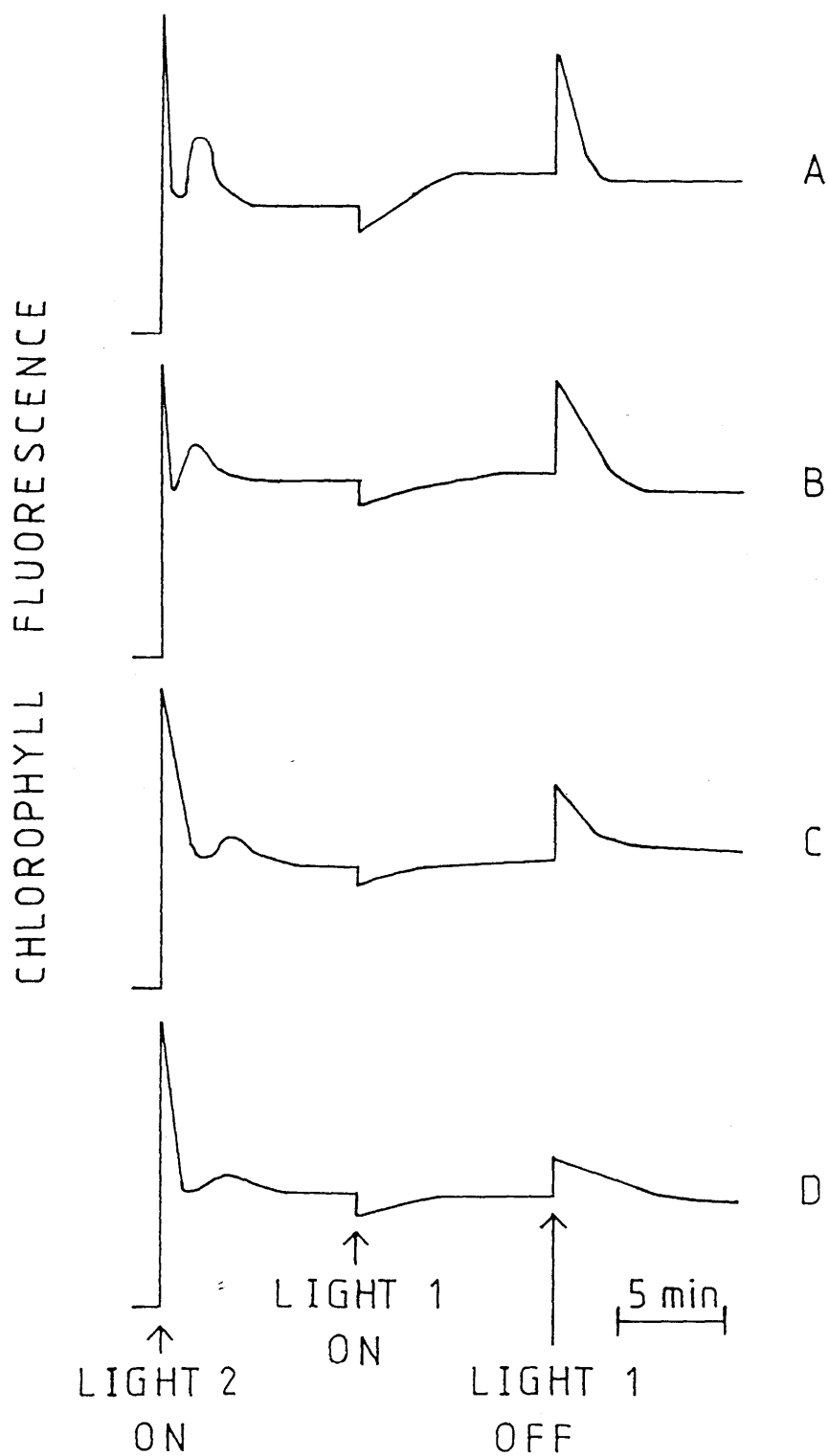


Figure 38. Legend as for Figure 37 except that 8 day old leaf pieces were used.

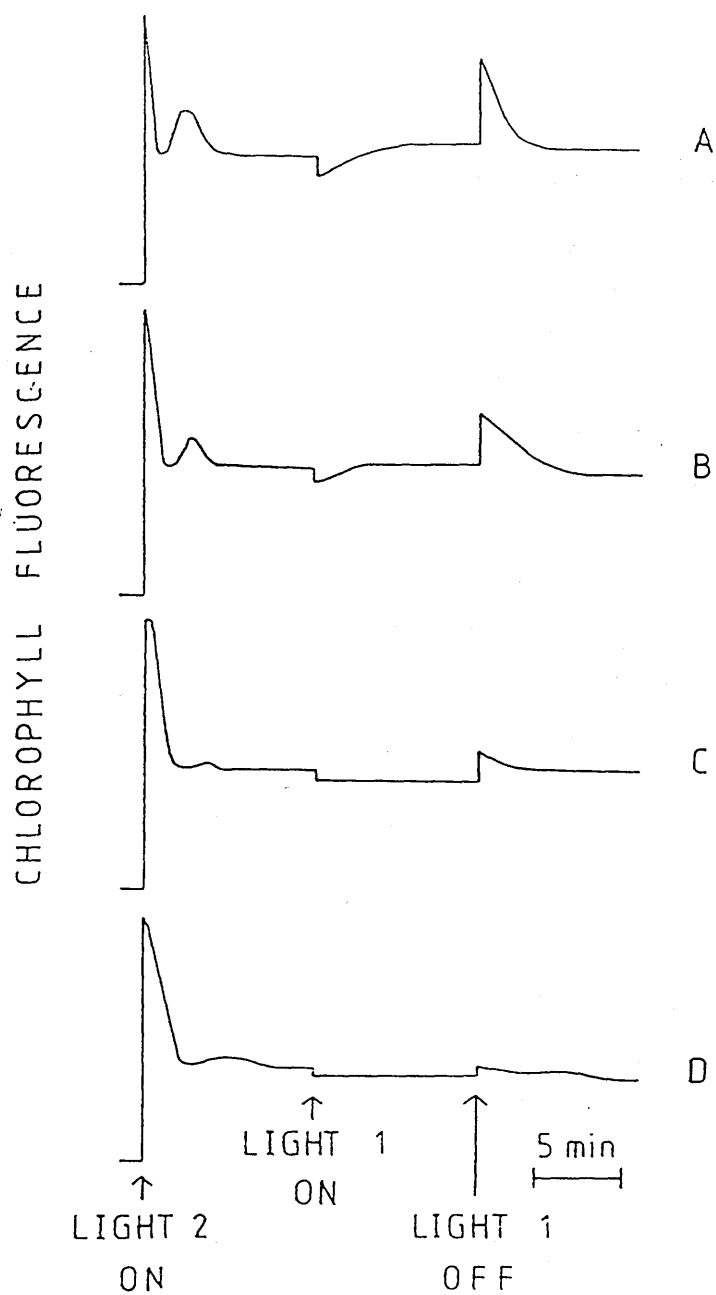


Figure 39. Legend as for Figure 37 except that 9 day old leaf pieces were used.

3.7.1 Discussion

The mechanisms underlying State 1 - State 2 transitions are thought to be complex. The redox state of plastoquinone, an electron carrier between PSII and PSI, is believed to serve as a detector of the imbalance of excitation between the two photosystems. Thus, over-excitation of PSII would result in plastoquinone becoming more reduced, whereas over-excitation of PSI would result in plastoquinone becoming more oxidised. The reduced state of plastoquinone is thought to activate a protein kinase enzyme in chloroplast thylakoid membranes, resulting in phosphorylation of the light-harvesting chlorophyll a/b - protein complex associated with PSII (LHC-II). Conversely, oxidation of plastoquinone is thought to activate a thylakoid phosphatase enzyme, resulting in dephosphorylation of LHC-II (Bennett, 1979; Horton and Black, 1980; Allen, Bennett, Steinback and Arntzen, 1981). A model of this hypothesis is shown in Figure 40.

Barber (1980, 1982) has postulated that the primary effect of LHC-II phosphorylation would be to insert charge into the appressed, electrically neutral regions of the thylakoid membrane (Figure 40). The consequent increase in charge density would upset the balance of attractive and repulsive electrostatic forces between adjacent membranes and cause movement of the charged LHC-II away from PSII towards PSI in the non-appressed regions of the thylakoid membrane. The pool of migratory LHC-II appears to be "free" LHC-II which is not bound tightly to PSII. This migration might be expected to change the light-absorption cross-section of the two photosystems in such a way that the fraction of energy initially

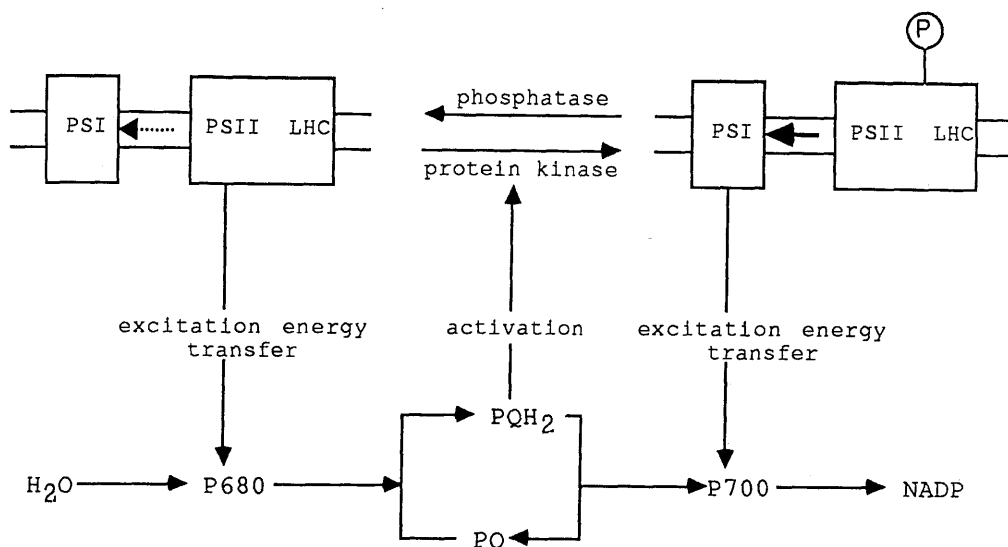


Figure 40. Regulation of distribution of excitation energy by redox state of plastoquinone (adapted from Allen (1983)). Preferential excitation of P680 relative to P700 causes reduction of plastoquinone, activation of protein kinase, phosphorylation of LHC and increased excitation energy transfer to P700. Preferential excitation of P700 relative to P680 causes oxidation of plastoquinone, inactivation of the kinase, dephosphorylation of LHC and increased excitation energy transfer to P680. PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; P680, reaction centre chlorophyll of PSII; P700, reaction centre chlorophyll of PSI; LHC, light-harvesting chlorophyll; P, phosphate group.

arriving at PSI would increase. Alternatively, the migration of LHC-II might be expected to allow greater interaction between PSII and PSI at the junction of the appressed and non-appressed regions of the thylakoid membrane, so that the "spillover" of energy from PSII to PSI would increase. Dephosphorylation of LHC-II would be expected to induce movement of LHC-II back to PSII, thus increasing the amount of energy into PSII (Figure 40).

Exactly how much of the regulation of excitation energy distribution is due to changes in light-absorption cross-section and how much is due to changes in spillover of energy is still in doubt. In isolated thylakoids, the background level of cations appears to be important. For example, at concentrations of Mg^{2+} below 5mM, the phosphorylation mechanism seems to involve a change in spillover as well as adjustments in the absorption cross-sections of PSI and PSII. However, at higher Mg^{2+} levels (above 5mM), phosphorylation of LHC-II seems to cause mainly changes in absorption cross-section (Telfer, Hodges and Barber, 1983). In mature leaves, regulation of excitation energy distribution appears to be fully accounted for by changes in absorption cross-section and does not appear to involve a significant change in spillover from PSII to PSI (Malkin, Telfer and Barber, 1986); Canaani and Malkin, 1984).

In this section, State 1 - State 2 transitions in control and salt-stressed barley leaf pieces were compared by over-exciting PSI and PSII in turn; any resultant change in the distribution of light energy between the two photosystems was then examined using modulated fluorimetry.

After control leaf pieces had been illuminated for 10 minutes with light which preferentially excited PSII (Light 2), State 2

was established wherein the distribution of light energy between the two photosystems was in favour of PSI. Upon adding Light 1 (which preferentially excited PSI), the immediate decrease in chlorophyll fluorescence indicated that the rate of electron transport through PSI had been increased by the addition of Light 1, with the result that Q had become more oxidised. Leaving on Light 1 for several minutes caused less light energy to be distributed to PSI and relatively more to PSII, resulting in a slow increase in chlorophyll fluorescence as Q became more reduced i.e. the photosynthetic system was now in State 1. On turning off the added PSI light, because the system was in State 1, the preferential excitation of PSII by Light 2 led to a more reduced state of Q and hence a very fast rise in the chlorophyll fluorescence signal. Chlorophyll fluorescence then fell rapidly to the steady-state level as light energy was redistributed in favour of PSI and the photosynthetic system returned to State 2.

In salt-stressed leaf pieces, turning on Light 1 induced a rise in chlorophyll fluorescence that was smaller and more gradual than in control leaf pieces. This suggests that less light energy was being redistributed in favour of PSII in the salt-stressed leaf pieces than in the control leaf pieces. There are two possible explanations for this salt effect. Firstly, salt may inhibit the initial transition from State 1 to State 2 so that State 2 is formed only partially or, secondly, salt may inhibit the transition from State 2 to State 1 but not the initial transition from State 1 to State 2. The former possibility seems the more likely since it would be consistent with the results of the light doubling experiments (Section 3.6) which showed that Q was in a more reduced state throughout the induction curves of salt-stressed leaf pieces; an inhibition in the transition from

State 1 to State 2 would be expected to increase PSII activity at the expense of PSI, leading to a more reduced state of Q. The slower rate of decline to the steady-state level in salt-stressed leaves supports the hypothesis that salt is inhibiting the transition from State 1 to State 2.

If salt caused only an inhibition in the transition from State 1 to State 2, the addition of Light 1 would be expected to increase the rate of non-cyclic electron transport through PSI and thus increase the oxidation of Q. However, when Light 1 was turned on, the immediate fall in chlorophyll fluorescence was not more pronounced in the salt-stressed leaf pieces than in the control leaf pieces. This might be explained by salt having caused an inhibition in electron transport between Q and PSI, with the result that the rate of electron transport was already maximal before the PSI light was turned on.

Taking the hypothesis of the underlying mechanisms of State 1 - State 2 transitions into account, there are two possible sites that salt may affect to inhibit the transition from State 1 to State 2. Firstly, salt may inhibit the ability of the plastoquinone to activate the protein kinase enzyme. Secondly, salt may inhibit the ability of the protein kinase enzyme to phosphorylate LHC-II.

3.8 The Effect of Increasing the External [NaCl] on the Emission of Low Temperature Chlorophyll Fluorescence

The results of the State 1 - State 2 transition experiments suggested that salt stress inhibits the transition from State 1 to State 2 in barley leaf pieces. Another technique which can be

used to examine changes in the distribution of excitation energy within the photosynthetic apparatus is to study the chlorophyll fluorescence emission spectrum of leaf pieces frozen in liquid nitrogen to 77K. These experiments were carried out in collaboration with Prof. N.R. Baker at the University of Essex.

At 20°C the chlorophyll fluorescence emission spectrum of higher plant chloroplasts has a large peak at about 680-695nm with a very small peak at about 740nm (Figure 41). Most of the emission in this spectrum is from PSII chlorophylls, with PSI chlorophyll molecules making only a very small contribution. However, freezing samples in liquid nitrogen to 77K reduces the thermal excitation of vibrational substates and reveals more details of the peaks in the spectrum. Thus the 77K spectrum has three peaks at about 685, 695 and 740nm (Figure 41). The 685nm band is attributed to both the light-harvesting chlorophyll-protein complex of PSII and to the core complex of PSII, whilst the 695nm band is attributed solely to pigments of the PSII core complex (Bose, 1982). The large 740nm band is generally considered to be emissions from PSI chlorophylls and these PSI chlorophylls are also thought to have a major emission in the 720nm region (Bose, 1982).

Since the chlorophyll fluorescence emission spectrum at 77K reveals more details of PSII chlorophyll fluorescence and has a much higher level of chlorophyll fluorescence associated with PSI, it can be of greater use than the 20°C emission spectrum for comparing the amount of excitation energy associated with each photosystem.

Before comparing the 77K chlorophyll fluorescence emission spectra of control and salt-stressed barley leaf pieces, the leaf pieces were exposed to one of two treatments.

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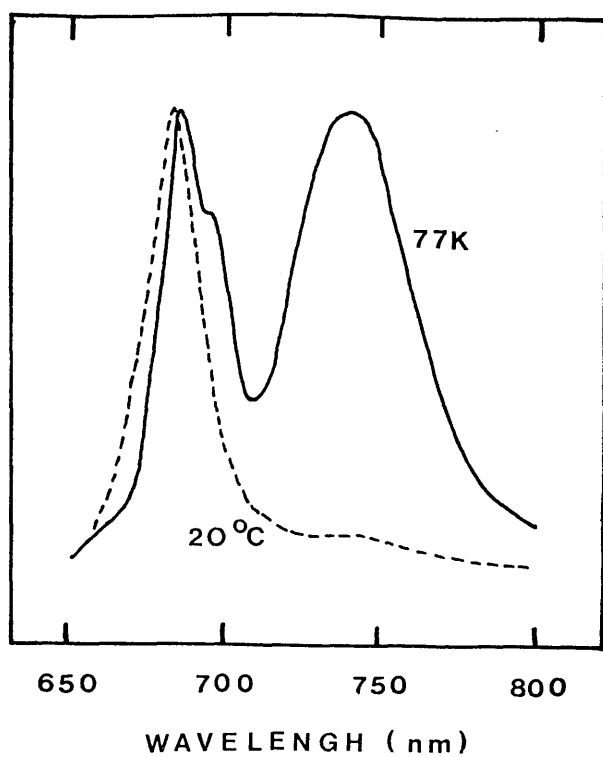


Figure 41. Typical chlorophyll fluorescence emission spectra of chloroplasts at 20°C (---) and 77K (—). (From Hipkins and Baker (1986)).

- 1) One set each of control and salt-stressed leaf pieces was dark-adapted for 10 minutes, frozen for 5 minutes and then illuminated. The resultant spectra showed the level of chlorophyll fluorescence associated with photosystems I and II when the leaf pieces were assumed to be in State 1.
- 2) A second set each of control and salt-stressed leaf pieces was pre-illuminated for 10 minutes, frozen for 5 minutes and then illuminated again. The resultant spectra showed the level of chlorophyll fluorescence associated with photosystems I and II when the leaves were in State 2.

Table XIII shows the ratio of chlorophyll fluorescence at 740nm (F740) over the chlorophyll fluorescence at 685nm (F685) for 8 day old control and salt-stressed leaf pieces. The table also shows the ratio of F740 to the chlorophyll fluorescence at 695nm (F695). Table XIV shows the percentage increase in these two ratios during a transition from State 1 to State 2 in control and salt-stressed leaf pieces.

On going from State 1 to State 2, the percentage increase in both F740/F685 and F740/F695 fell as the external [NaCl] was increased.

3.8.1 Discussion

After salt-stressed leaf pieces had been exposed to a period of illumination that was expected to induce the photosynthetic system into State 2, the ratio of PSI chlorophyll fluorescence to PSII chlorophyll fluorescence was much lower than that of control leaf pieces. A possible explanation for this is that salt inhibits the transition from State 1 to State 2 with the result that, after the period of pre-illumination, the level of chlorophyll

T A B L E XIV

The Percentage Increase in F740/F685 and F740/F695 During a
Transition From State 1 to State 2 in Control and Salt-Stressed 8 Day
Old Leaf Pieces

[NaCl]mM	% Increase In F740/F685	% Increase In F740/F695
Control	45	50
250	31	48
400	16	24
600	-17	-15

T A B L E X I I I

F740/F685 and F740/F695 Ratios for 8 Day Old Control and Salt-Stressed Barley Leaf Pieces

The leaf pieces were illuminated with 27Wm^{-2} of 440nm irradiation. Measurements of chlorophyll fluorescence emission from 650-800nm were made through a scanning high radiance monochromator. Chlorophyll fluorescence was detected by a photomultiplier.

Sample	F740/F685	F740/F695
Control (State 1)	3.1	3.0
Control (State 2)	4.5	4.5
250mM NaCl (State 1)	2.6	2.3
250mM NaCl (State 2)	3.4	3.4
400mM NaCl (State 1)	2.5	2.5
400mM NaCl (State 2)	2.9	3.1
600mM NaCl (State 1)	4.6	4.0
600mM NaCl (State 2)	3.8	3.4

fluorescence associated with PSII is higher compared to the control while the level of chlorophyll fluorescence associated with PSI is lower.

However, it should be emphasised that these results have several limitations. Firstly, there was no opportunity to replicate these experiments. Secondly, since no internal standard was used, it cannot be stated with certainty that the changes in F740/F685 or F740/F695 were due to an increase in F685 or F695, and a corresponding decrease in F740. Finally, on freezing to 77K, samples were often not optically homogeneous due to cracking of ice.

Despite these problems, the results of this experiment appear to support those of the State 1 - State 2 transition experiments (Section 3.7) in suggesting that salt inhibits the transition from State 1 to State 2.

3.9 Summary of Chapter 3

NaCl was found to inhibit the growth and to reduce the water potential of barley leaves. At high external NaCl concentrations, the leaves contained high concentrations of Na^+ ions, possibly as a result of root damage.

The major effect of NaCl on photosynthesis in barley seemed to be to inhibit the transition from State 1 to State 2 under conditions of over-excitation of PSII. This caused an increase in the activity of PSII at the expense of PSI and led to a more reduced state of Q and an inhibition in oxygen evolution. Salt stress also appeared to inhibit one or more reactions of the RPP pathway, leading to a delay in the time taken to reach the S and M chlorophyll fluorescence levels, and a decrease in the height of the M peak.

OSMOTIC STRESS

4.1 Selection of an Impermeable Solute

NaCl has been found to cause loss of water from barley leaves (Section 3.2.2). In addition, however, high concentrations of Na^+ ions have been found inside the leaves of severely stressed barley (Section 3.2.3) so that toxic effects of NaCl may also play an important role. One way to determine whether an osmotic or a toxic effect is the major component of salt stress in barley is to compare the effects of NaCl-stressing solutions with isotonic solutions of an impermeable substance. If the salt injury is simply osmotic, the two solutes should produce the same injury at the same osmotic potential. If, however, some toxic effect of NaCl is involved, a NaCl solution should cause a greater salt injury than that of a solution of an impermeable substance at the same osmotic potential.

Mannitol was chosen as the impermeable substance with which to stress barley leaves because it has been shown to be an appropriate agent for inducing water stress in green plants (Hoad, 1975; Mizrahi, Blumenfeld and Richmond, 1970). The concentrations of mannitol required to give mannitol-stressing solutions of the same osmotic potential (O.P.) as the salt-stressing solutions are shown in Table XV.

T A B L E X V

Osmotic Potentials of Salt-Stressing Solutions and the Corresponding
Concentrations of Mannitol Required to Give Mannitol-Stressing
Solutions of the Same Osmotic Potential

Five replicates of each mannitol solution were tested by the cryoscopic method described in Section 2.4, and the mean of the Knauer readings obtained was used to read off the osmotic potential of the mannitol solution from the calibration graph.

Solution	Osmotic Potential (MPa)	[Mannitol] required
Nutrient soln. only	- 0.072	0
Nutrients + 250mM NaCl	- 1.138	476mM
Nutrients + 400mM NaCl	- 1.743	708mM
Nutrients + 600mM NaCl	- 2.594	1037mM

4.2 The Effect of Stressing Barley Leaves with Mannitol Solutions of the Same Osmotic Potential as the Salt-Stress Solutions

4.2.1 The Effect of Increasing the External [Mannitol] on the Height of the Primary Leaves of Barley

The heights of the primary leaves of control and mannitol-stressed plants were measured after 7 and 14 days. Figure 42 compares these results with the results obtained previously for the salt-stress solutions (Section 3.2.1).

The height of the primary leaves decreased as the external [mannitol] increased. This decrease in height was more pronounced than the decrease in height caused by NaCl solutions of the same osmotic potential, especially after 14 days when each set of mannitol-stressed leaves showed almost no growth after the first period of 7 days.

4.2.2 The Effect of Increasing the External [Mannitol] on the Water Potential of Barley Leaves

To determine whether mannitol does subject barley to osmotic stress, the water potentials of 7, 8 and 9 day old control and mannitol-stressed leaves were measured. Figure 43 compares these results with the results obtained previously for the salt-stress solutions (Section 3.2.2).

For 7, 8 and 9 day old leaves, increasing the external [mannitol] caused a much larger fall in the leaf water potential than NaCl solutions of the same osmotic potential. However, the manner in which the leaf water potential changed as the external [mannitol] increased was similar to the way in which the leaf

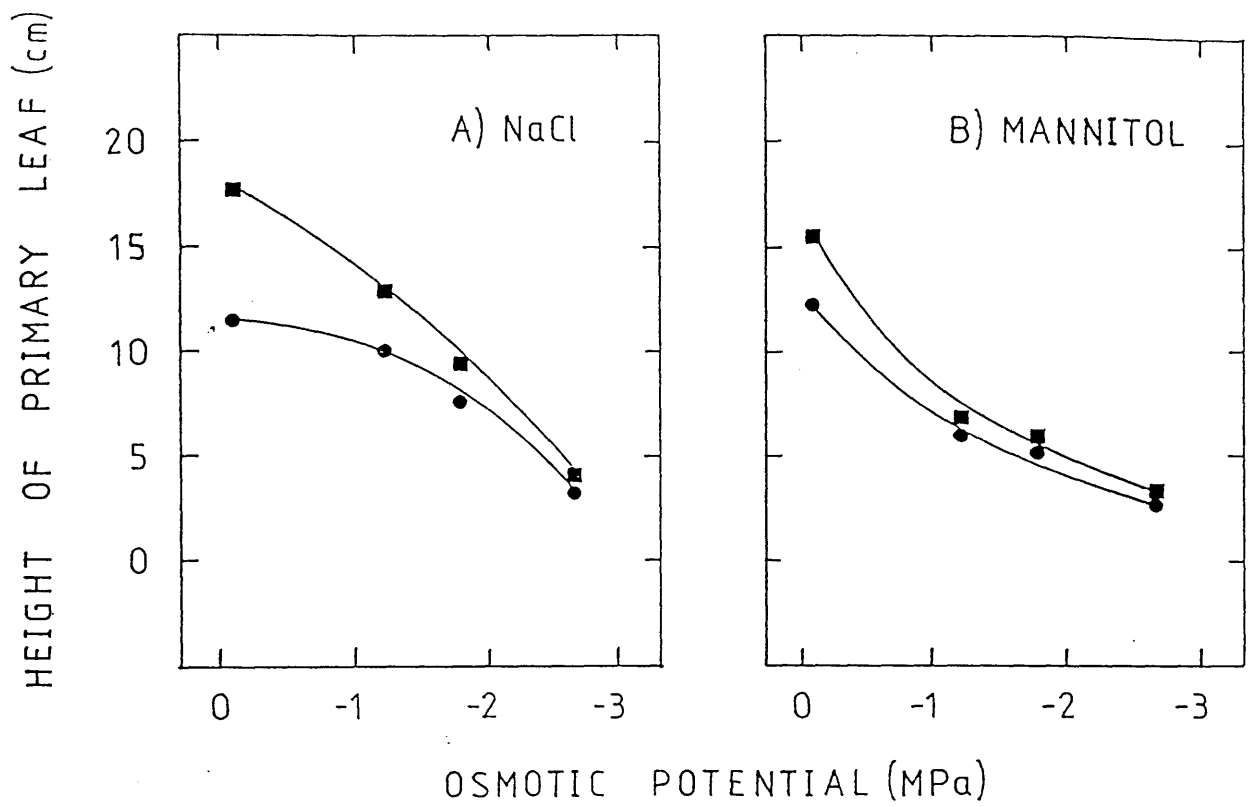


Figure 42. The effect of increasing the external A) [NaCl] and B) [mannitol] on the height of the primary leaves of barley measured after 7 (● — ●) and 14 (■ — ■) days. Results indicate the mean \pm S.E.M. for 20 determinations. Standard error bars were within the symbol size.

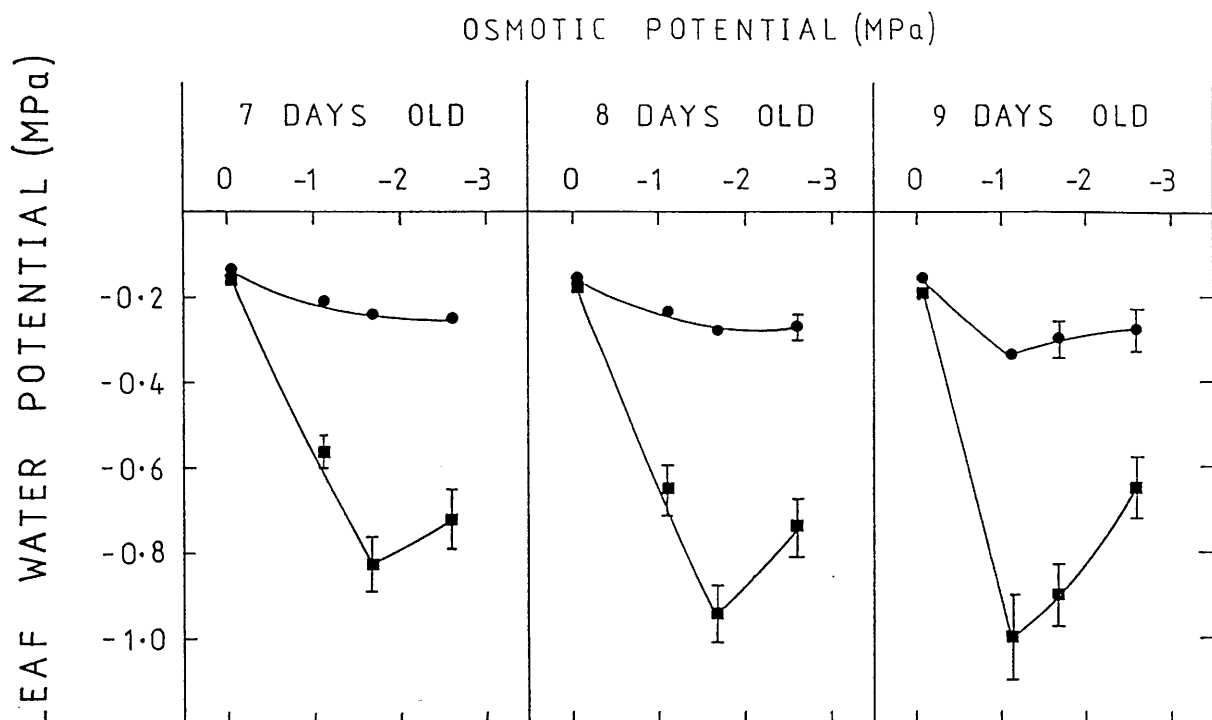


Figure 43. The effect on leaf water potential of stressing barley leaves with NaCl (● — ●) and mannitol (■ — ■) solutions of the same osmotic potential. Each point represents the mean \pm S.E.M. of 10 determinations. Where standard error bars are not shown, errors were within the symbol size.

water potential changed as the external [NaCl] increased. Initially, the leaf water potential declined as the external [mannitol] increased but, at the highest mannitol concentrations, the leaf water potential rose.

4.2.3 The Effect of Increasing the External [Mannitol] on the Internal Mannitol Content of Barley Leaf Pieces

As already explained in Section 3.2.4 for NaCl, a possible reason for the rise in the water potential of leaves stressed by high external mannitol concentrations is that the roots of barley become damaged at high external mannitol concentrations, resulting in high concentrations of mannitol inside the leaves. To try to determine if this explanation is correct, the content of mannitol inside stressed leaf pieces was estimated by gas-liquid chromatography.

Table XVI shows the internal mannitol content of the stressed leaf pieces. It can be seen that leaf pieces stressed by the highest external mannitol concentration had a very high internal mannitol content.

4.2.4 Discussion

Stressing leaves with mannitol solutions had a more severe effect on the growth and water potential of barley leaves than had NaCl solutions of the same osmotic potential. Leaves which showed a rise in water potential compared to leaves stressed by lower mannitol concentrations also had a much higher internal mannitol content; this supports the hypothesis that at high external mannitol concentrations, barley roots become damaged

T A B L E X V I

Internal Mannitol Contents of Barley Leaf Pieces Stressed by 476mM Mannitol (O.P. = 250mM NaCl), 708mM Mannitol (O.P. = 400mM NaCl) and 1037mM Mannitol (O.P. = 600mM NaCl)

Results indicate the mean \pm S.E.M. for 3 determinations.

External [Mannitol]mM	Surface % Contamination	Internal Mannitol Content (mM Tissue Water)		
		7 Days	8 Days	9 Days
Control	-	0.3 \pm 0.13	0.6 \pm 0.18	0.2 \pm 0.08
476	13	53 \pm 6.0	71 \pm 9.3	53 \pm 0.3
708	-	51 \pm 6.1	114 \pm 11.6	102 \pm 10.5
1037	14	293 \pm 60.3	320 \pm 131.8	317 \pm 32.6

and allow the entry of high concentrations of mannitol.

Uptake of mannitol in considerable amounts into the shoots of intact barley plants has been observed previously under conditions where the mannitol is being used at high concentrations for long-term experiments (Groenewegen and Mills, 1960). Under such circumstances, toxic effects of a high mannitol content inside the leaf have been distinguished from purely osmotic effects caused by mannitol outside the leaf (Resnik, 1970).

When stressed with mannitol solutions of the same osmotic potential as the salt solutions, barley leaf pieces contained high concentrations of mannitol. It would therefore be difficult to determine in future experiments whether any effects of mannitol stress were due purely to osmotic stress or whether other physiological effects of mannitol were involved. To try to avoid this problem, it was decided not to distinguish between toxic and osmotic effects of NaCl by comparing the effects of NaCl and mannitol solutions of the same osmotic potential. Instead, it was decided to compare the effects of NaCl and mannitol solutions which were known to induce the same leaf water potential after the same length of time. Since these NaCl and mannitol solutions must then be causing exactly the same level of water stress, it should be possible to distinguish between toxic and osmotic effects of NaCl. It was thought that this might be a better method because, from Figure 43, the external mannitol concentrations required to induce the same leaf water potentials as the salt-stress solutions should be lower than those that were required to give the same osmotic potentials as the salt-stress solutions. As a result, it was hoped that the internal mannitol content of the stressed leaf pieces would also be lower.

4.3 The Effect of Stressing Barley Leaves with Mannitol Concentrations in the Range 0-400mM

4.3.1 Effect on the Water Potential

Figure 44 shows the water potentials of 7, 8 and 9 day old barley leaves stressed with 0, 100mM mannitol (O.P. = -0.260MPa), 200mM mannitol (O.P. = -0.510MPa), 300mM mannitol (O.P. = -0.730MPa) and 400mM mannitol (O.P. = -0.980MPa). This figure also compares these results with the results obtained previously for the salt-stress solutions (Section 3.2.2).

It can be seen that in 8 day old barley leaves, a 300mM mannitol solution induced the same leaf water potential as a 400mM NaCl solution.

4.3.2 Effect on the Internal Mannitol Content

Table XVII shows the internal mannitol content of 7, 8 and 9 day old leaf pieces stressed with 0-400mM mannitol.

The results show that 8 day old leaf pieces stressed by a 300mM mannitol solution contained a relatively low internal mannitol content compared to the leaves which were stressed with 400 to 1037mM mannitol solutions.

4.3.3 Discussion

As a result of these experiments, it was decided to distinguish between toxic and osmotic effects of NaCl by stressing 8 day old barley leaves with 300mM mannitol solutions and then comparing these results with the effect of stressing 8

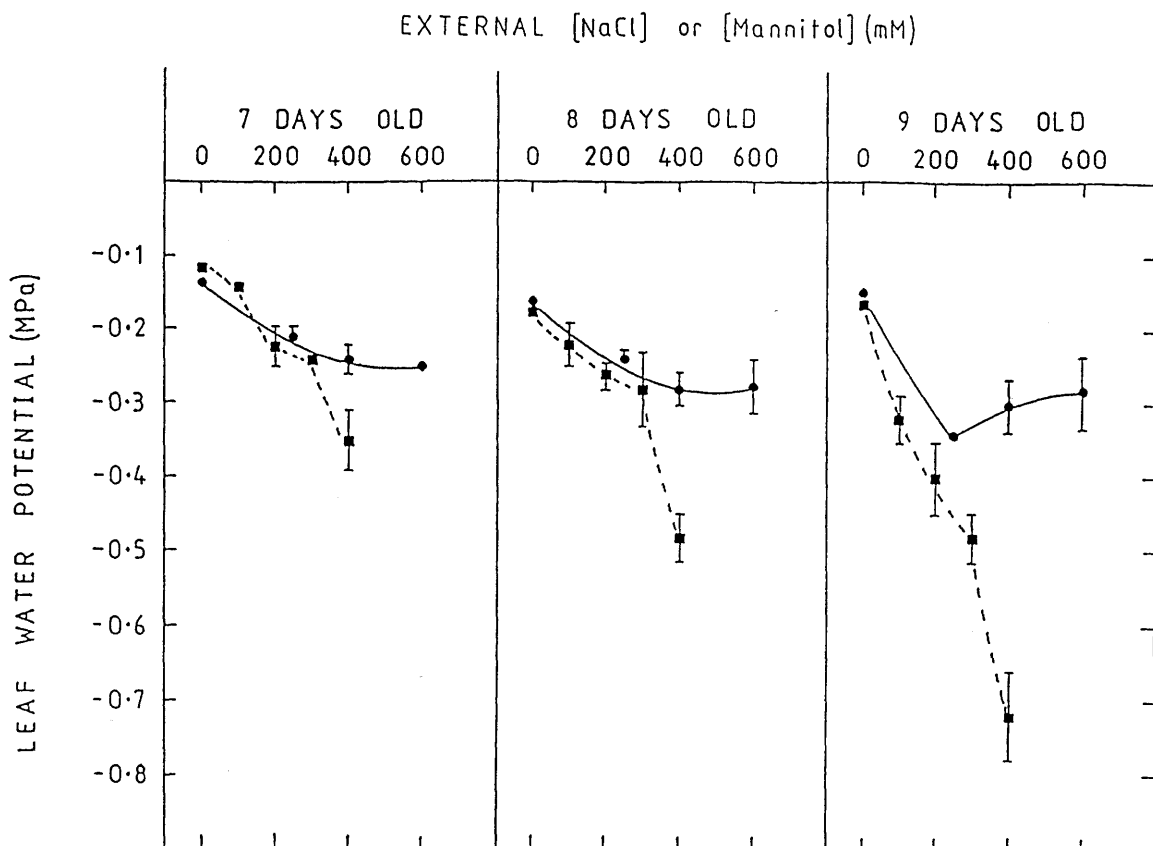


Figure 44. Water potentials of 7, 8 and 9 day old barley leaves stressed with NaCl (●—●) or mannitol (■---■) solutions. Each point represents the mean \pm S.E.M. of 10 determinations. Where standard error bars are not shown, errors were within the symbol size.

T A B L E XVII

Internal Mannitol Content of Leaf Pieces Stressed by 0, 100mM Mannitol
(O.P. = -0.260MPa), 200mM Mannitol (O.P. = -0.510MPa), 300mM
Mannitol (O.P. = -0.730MPa) and 400mM Mannitol (O.P. = -0.980MPa)

Results indicate the mean \pm S.E.M. for 3 determinations.

External [Mannitol]mM	% Surface Contamination	Internal Mannitol Content (mm Tissue Water)		
		7 Days	8 Days	9 Days
Control	-	0.3 \pm 0.13	0.6 \pm 0.18	0.2 \pm 0.08
100	8	15 \pm 1.5	17 \pm 0.6	21 \pm 3.5
200	-	31 \pm 1.2	21 \pm 2.5	27 \pm 3.9
300	13	40 \pm 3.2	32 \pm 3.5	30 \pm 0.7
400	-	68 \pm 2.0	72 \pm 3.5	38 \pm 1.8

day old barley leaves with 400mM NaCl.

4.4 Comparison of the Effect of Stressing Barley Leaves with Mannitol (300mM) and NaCl (400mM) Solutions Which Induce the Same Leaf Water Potential

4.4.1 Effect on the Height of the Primary Leaf

Table XVIII compares the heights of 8 and 16 day old barley leaves stressed with 300mM mannitol and 400mM NaCl. This table also shows the effect of stressing barley leaves with 100mM mannitol as an intermediate stressing concentration.

For 8 day old leaves, stressing with 100mM mannitol had almost no effect on height. Stressing with 300mM mannitol caused a decrease in the height of the leaves but this decrease was not as large as that caused by 400mM NaCl.

For 16 day old leaves, the effect of all three stressing solutions was more pronounced. Again, stressing with 400mM NaCl had a larger effect than 300mM mannitol.

4.4.2 Effect on Stomatal Shape

The dimensions of the stomatal pores of barley leaves stressed by 100mM mannitol, 300mM mannitol and 400mM NaCl were measured using Xantopren impressions as described previously (Section 3.4.1). Results are shown in Table XIX. The rates of CO₂ intake into each set of stomata are shown in Table XX.

As observed previously, the rate of CO₂ intake into each set of leaf pieces from the chamber was 100 times faster than the

T A B L E XVIII

The Effect of 100mM Mannitol, 300mM Mannitol and 400mM NaCl on the
Height of the Primary Leaves of Barley

Results indicate the mean \pm S.E.M. for 20 determinations.

Stressing Solution	Height Of Primary Leaf (cm)	
	8 Days Old	16 Days Old
Control	10.4 \pm 0.18	14.6 \pm 0.28
100mM Mannitol	10.2 \pm 0.42	12.8 \pm 0.18
300mM Mannitol	9.0 \pm 0.19	11.6 \pm 0.20
400mM NaCl	7.1 \pm 0.13	8.1 \pm 0.24

T A B L E XIX

The Length and Width of the Stomatal Pores of 8 Day Old Barley Stressed by 100mM Mannitol, 300mM Mannitol and 400mM NaCl

Measurements of the stomatal pores were carried out after exposing control and stressed barley to the atmosphere inside the growth room and leaf chamber. These measurements were made using Xantopren impressions. The results indicate the mean \pm S.E.M. for 30 determinations.

Stressing Solution	Length or Width of Stomatal Pore (μ m)		
		Growth Room	Leaf Chamber
Control	Length	22 \pm 0.9	26 \pm 0.5
	Width	2.4 \pm 0.21	0.7 \pm 0.10
100mM Mannitol	Length	22 \pm 0.8	27 \pm 0.7
	Width	2.2 \pm 0.17	0.6 \pm 0.19
300mM Mannitol	Length	24 \pm 0.7	25 \pm 0.7
	Width	1.5 \pm 0.19	0.9 \pm 0.20
400mM NaCl	Length	27 \pm 0.7	27 \pm 0.5
	Width	1.0 \pm 0.10	0.5 \pm 0.11

T A B L E XX

Rates of Carbon Dioxide Intake by 8 Day Old Barley Stressed by 100mM Mannitol, 300mM Mannitol and 400mM NaCl

Rates of carbon dioxide intake were calculated using the mean values for stomatal dimensions obtained in Table XIX. Results are given for leaves from the growth room and for leaf pieces from the leaf chamber.

Stressing Solution	Rate of Carbon Dioxide Intake (mg CO ₂).cm ⁻² . s ⁻¹)	
	Growth Room	Leaf Chamber
Control	6.1 x 10 ⁻⁵	6.3 x 10 ⁻³
100mM Mannitol	5.9 x 10 ⁻⁵	5.9 x 10 ⁻³
300mM Mannitol	5.4 x 10 ⁻⁵	7.4 x 10 ⁻³
400mM NaCl	4.8 x 10 ⁻⁵	5.7 x 10 ⁻³

rate of CO₂ intake into any of the leaf sets from the growth room. In addition, the stomata from leaves stressed by 400mM NaCl were again less open in the growth room than those from control leaves, with the result that their rate of CO₂ intake was lower than that of control leaves. Stomata from leaves stressed by 300mM mannitol were less open in the growth room than control leaves but were not as closed as stomata from 400mM NaCl-stressed leaves. Similarly, the rate of CO₂ intake by 300mM mannitol-stressed leaves in the growth room was lower than that of the control, but was not as low as that of 400mM NaCl-stressed leaves. Since leaves stressed by 300mM mannitol and by 400mM NaCl showed similar values of leaf water potential, the values for their stomatal dimensions and rates of CO₂ intake might have been expected to be more similar.

4.4.3 Effect on Chlorophyll Content

Table XXI shows the chlorophyll content of 8 day old barley leaf pieces stressed by 100mM mannitol, 300mM mannitol and 400mM NaCl.

There was very little difference in the chlorophyll content of control leaf pieces and leaf pieces stressed by mannitol concentrations up to 300mM. However, leaf pieces stressed by 400mM NaCl showed a 17% decrease in chlorophyll content.

4.4.4 Effect on Oxygen Evolution

Table XXII shows the steady-state rates of oxygen evolution of 8 day old barley leaf pieces stressed by 100mM mannitol, 300mM mannitol and 400mM NaCl.

T A B L E XXI

The Effect of 100mM Mannitol, 300mM Mannitol and 400mM NaCl on the
Chlorophyll Content of 8 Day Old Barley Leaf Pieces

Results indicate the mean \pm S.E.M. for 7 determinations.

Stressing Solution	μg Chlorophyll / 100mg Wet Weight
Control	222 \pm 8.2
100mM Mannitol	211 \pm 7.7
300mM Mannitol	216 \pm 7.9
400mM NaCl	185 \pm 8.0

T A B L E XXII

The Effect of 100mM Mannitol, 300mM Mannitol and 400mM NaCl on the
Steady-State Rates of Oxygen Evolution by 8 Day Old Barley Leaf
Pieces

Results indicate the mean \pm S.E.M. for 7 determinations.

Stressing Solution	O ₂ Evolution Rate ($\mu\text{moles O}_2(\text{mg chl})^{-1}\text{h}^{-1}$)
Control	225 \pm 23.1
100mM Mannitol	207 \pm 17.3
300mM Mannitol	170 \pm 16.0
400mM NaCl	112 \pm 9.5

There was very little difference in the oxygen evolution rates of control leaf pieces and leaf pieces stressed by 100mM mannitol. Leaf pieces stressed by 300mM mannitol showed a decrease (24%) in the oxygen evolution rate but this decrease was not as large as that caused by 400mM NaCl (50%).

4.4.5 Effect on Chlorophyll Fluorescence Induction Kinetics

The most obvious difference between the Kautsky chlorophyll fluorescence transients of 8 day old control barley leaf pieces and leaf pieces stressed by 100mM mannitol, 300mM mannitol and 400mM NaCl was the height of the S-M transient (Table XXIII). All three stressing solutions caused a severe decrease in the height of the S-M transient, with 400mM NaCl having the largest effect.

4.4.6 Effect on the Redox State of Q During Kautsky Transients : "Light Doubling"

The effect of 100mM mannitol, 300mM mannitol and 400mM NaCl on the redox state of Q during Kautsky transients in 8 day old barley leaf pieces was determined using the "light doubling" technique described in Sections 2.14.1 and 3.6. Results are shown in Table XXIV.

Leaves stressed by 100mM mannitol showed similar results to the control leaves : there was no clear difference in the values of % q_Q or in the time taken to reach the S and M chlorophyll fluorescence levels. Leaves stressed by 300mM mannitol showed a slight tendency for Q to be more reduced during P to S. In contrast, and as observed previously in Section 3.6, leaves

T A B L E XXIII

The Effect of 100mM Mannitol, 300mM Mannitol and 400mM NaCl on the
Height of the S-M Chlorophyll Fluorescence Transient, Relative to the
Height of the P Peak, in 8 Day Old Barley Leaf Pieces

Measurement of S-M was carried out as described in Section 3.5.
Results indicate the mean \pm S.E.M. for 16 determinations.

Stressing Solution	Height of the S-M Chlorophyll Fluorescence Transient, Relative to the Height of the P Peak (Relative Units)
Control	13.5 \pm 1.06
100mM Mannitol	8.6 \pm 1.35
300mM Mannitol	5.7 \pm 1.20
400mM NaCl	2.2 \pm 0.76

T A B L E XXIV

Measurement of % q_Q at 10 Second Intervals Along the Chlorophyll
Fluorescence Induction Curve of 8 Day Old Leaf Pieces Stressed by
100mM Mannitol, 300mM Mannitol and 400mM NaCl

S and M indicate the time at which these chlorophyll fluorescence levels appear at each salinity. T was measured after 6 minutes. Results indicate the mean \pm S.E.M. for 9 determinations.

Seconds After Illum.	% q_Q			
	Control	100mM Mannitol	300mM Mannitol	400mM NaCl
10	14 \pm 2.4	14 \pm 1.9	13 \pm 1.3	7 \pm 0.9
20	21 \pm 3.8	20 \pm 2.5	16 \pm 1.2	8 \pm 1.0
30	26 \pm 4.5	27 \pm 4.1	21 \pm 1.6	10 \pm 1.1
40	S 30 \pm 4.6	31 \pm 4.4	26 \pm 1.8	13 \pm 1.8
50	31 \pm 4.6	S 33 \pm 4.6	S 30 \pm 3.0	16 \pm 2.6
60	32 \pm 4.9	33 \pm 4.6	36 \pm 3.8	21 \pm 3.4
70	28 \pm 3.0	29 \pm 3.1	40 \pm 4.4	24 \pm 4.5
80	22 \pm 3.2	28 \pm 4.1	38 \pm 4.8	S 27 \pm 5.4
90	17 \pm 2.8	26 \pm 3.3	34 \pm 4.0	28 \pm 5.2
100	M 16 \pm 2.5	25 \pm 2.8	31 \pm 4.0	29 \pm 4.9
110	17 \pm 2.7	M 26 \pm 2.1	M 31 \pm 3.4	28 \pm 4.8
120	25 \pm 5.8	29 \pm 1.7	32 \pm 3.1	26 \pm 4.2
130	25 \pm 3.8	31 \pm 1.6	34 \pm 3.2	M 26 \pm 4.1
140	27 \pm 4.3	36 \pm 2.5	36 \pm 3.6	26 \pm 4.4
150	33 \pm 3.4	38 \pm 2.8	40 \pm 3.4	31 \pm 4.7
160	36 \pm 3.4	40 \pm 2.7	41 \pm 4.0	33 \pm 5.0
170	37 \pm 3.3	41 \pm 2.8	44 \pm 4.1	32 \pm 4.9
180	38 \pm 3.3	41 \pm 2.6	44 \pm 3.6	34 \pm 5.3
190	38 \pm 3.3	41 \pm 2.5	44 \pm 3.0	34 \pm 5.5
360	T 42 \pm 3.9	T 44 \pm 2.7	T 47 \pm 3.1	T 39 \pm 6.3

stressed by 400mM NaCl had much lower values for % q_Q during the P to S phase, and showed a delay in the time taken to reach both the S and M chlorophyll fluorescence levels.

4.4.7 Effect on State 1 - State 2 Transitions

The effect of 100mM mannitol, 300mM mannitol and 400mM NaCl on State 1 - State 2 transitions in 8 day old barley leaf pieces was determined as described previously in Sections 2.14.2 and 3.7. Figure 45 shows typical State 1 - State 2 chlorophyll fluorescence curves for the control and stressed leaf pieces.

Leaf pieces stressed by 100mM and 300mM mannitol solutions showed similar State 1 - State 2 chlorophyll fluorescence curves to control leaf pieces : there was no clear difference in the nature of the slow chlorophyll fluorescence increase when Light 1 was turned on, in the immediate rise in chlorophyll fluorescence when Light 1 was turned off, or in the rate of decline of chlorophyll fluorescence to the steady-state level. In contrast, and as observed previously in Section 3.7, leaves stressed by 400mM NaCl showed a more gradual and smaller increase in chlorophyll fluorescence when Light 1 was turned on, a reduction in the height of the immediate chlorophyll fluorescence signal when Light 1 was turned off and a decrease in the rate of decline of the chlorophyll fluorescence signal to the steady-state level.

4.4.8 Discussion

A summary of the effect of stressing 8 day old barley leaves with 100mM mannitol, 300mM mannitol and 400mM NaCl is shown in

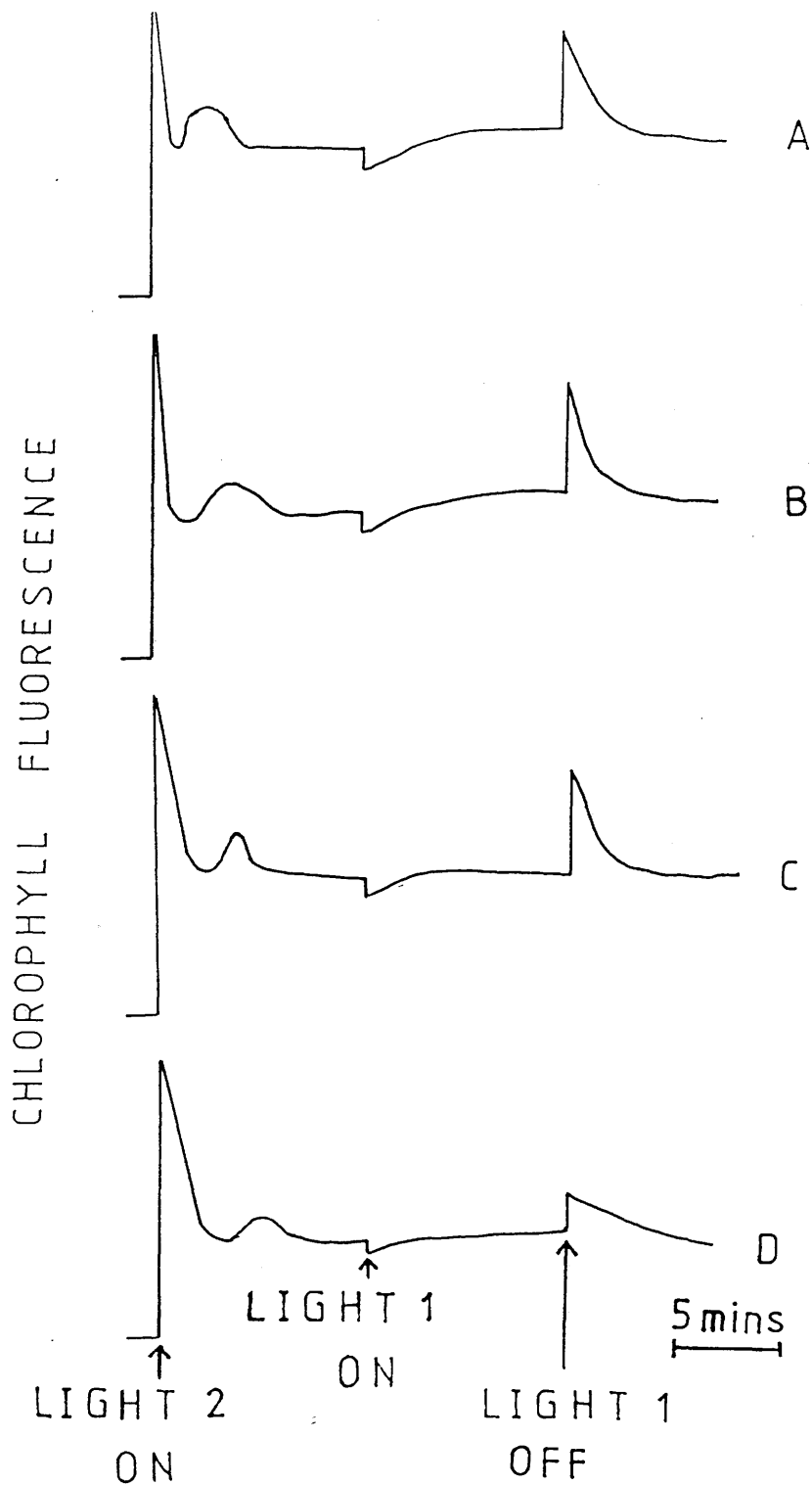


Figure 45. Typical State 1 - State 2 chlorophyll fluorescence curves for 8 day old control and stressed leaves. A) Control; B) 100mM mannitol; C) 300mM mannitol; D) 400mM NaCl. Each curve is representative of an experiment which was replicated at least five times.

Table XXV.

Stressing leaves with 400mM NaCl had a greater effect than 300mM mannitol on all the parameters studied. Since these two stressing solutions were found to have the same leaf water potential (Section 4.3.1), the greater effect of 400mM NaCl seems to indicate that the effect of NaCl stress on barley leaves is not purely osmotic; toxic effects of NaCl also appear to be involved.

Toxic effects of NaCl seem to be the major factor affecting chlorophyll content, $\%q_Q$ and State 1 - State 2 transitions, since stressing with mannitol has almost no effect on these parameters. However, osmotic effects play much more of a role in inhibiting growth and oxygen evolution, and especially in decreasing the height of the M peak.

T A B L E XXV

Summary of the Effect of 100mM Mannitol, 300mM Mannitol and 400mM NaCl on 8 Day Old Barley Leaves

The number of dashes indicates the extent of inhibition caused by each stressing solution. "O" indicates no effect.

Parameter	Stressing Solution		
	100mM Mannitol	300mM Mannitol	400mM NaCl
Height of primary leaf	-	--	-----
Chlorophyll content	O	O	-
Oxygen evolution	O	--	-----
Height of S-M	-----	-----	-----
% q_Q	O	O	-----
State 1 - State 2 transitions	O	O	---

THE USE OF PROTOPLASTS TO INVESTIGATE
SALT AND OSMOTIC STRESS

5.1 Protoplasts as an Experimental System to Study Photosynthesis

Chapters 3 and 4 have discussed the effects of salt and osmotic stress on barley leaves. The use of leaves enabled the photosynthetic apparatus of control and stressed plants to be compared in an intact system. Photosynthetic studies on leaves are often complemented by studies on protoplasts (cells lacking cell walls but with an intact plasmalemma) because protoplast metabolism is assumed to be comparable to the metabolism of whole cells in leaves (Pallett, Rees, Fitzsimons and Cobb, 1986). In addition, protoplasts are more amenable to experimental manipulation, particularly in studies of electron transport; unlike leaves, protoplasts have no epidermal barriers preventing the rapid penetration of electron transport inhibitors, acceptors or uncouplers.

The inhibitor 3- (3,4- dichlorophenyl)-1, 1- dimethylurea (DCMU), which blocks electron flow from Q to plastoquinone (Duysens and Sweers, 1963), causes a biphasic relaxation of chlorophyll fluorescence when added to a stirred suspension of protoplasts (Quick and Horton, 1984b), intact chloroplasts and algae (Krause et al., 1982). This biphasic increase in chlorophyll fluorescence is shown for intact chloroplasts in Figure 46. Krause et al. (1982) attributed the fast phase (R_f) to relaxation of q_Q , and the slow phase (R_s) to relaxation of q_e . Measurement of R_f and R_s , therefore, gives an estimate of how

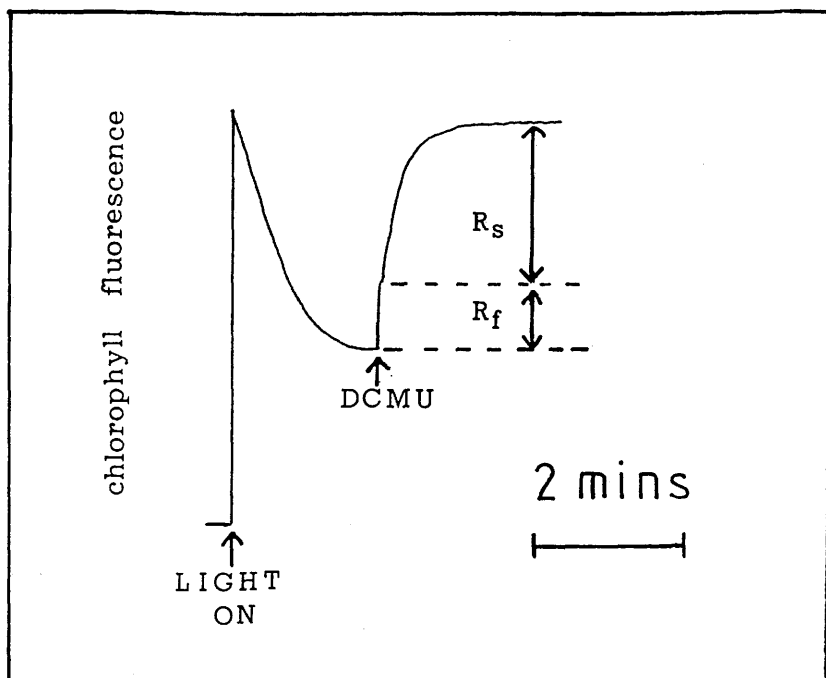


Figure 46. The effect of addition of DCMU ($4 \times 10^{-5} \text{M}$) on chlorophyll fluorescence in isolated, intact spinach chloroplasts. R_f and R_s represent the fast and slow phases of the increase in chlorophyll fluorescence, respectively, on addition of DCMU. (From Krause et al. (1982)).

much of the chlorophyll fluorescence quenching in an experimental system is due to q_Q and how much is due to q_e .

The extent to which q_Q is affected by salt and osmotic stress has been studied by light doubling in leaves (Sections 3.6 and 4.4.6). However, none of the other quenching mechanisms (Section 1.8.1) has been investigated so far. One method which might be used to examine the effect of salt and osmotic stress on q_e would be the addition of DCMU to barley protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol and 400mM NaCl. However, the values of q_e obtained by this method would only be useful if the protoplasts could be isolated from the control and stressed leaves without being damaged. Before adding DCMU, therefore, it is important to estimate the percentage intactness of the protoplast preparations, and to compare the photosynthetic parameters of the protoplasts with those of the corresponding control and stressed leaves from which the protoplasts were isolated.

5.1.1 Intactness

The percentage intactness of barley protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol and 400mM NaCl was tested at the end of the isolation procedure using Evans Blue stain (Section 2.9.3). Results are shown in Table XXVI.

Protoplasts isolated from the control and mannitol-stressed leaves had a high level of intactness at the end of the isolation procedure. In contrast, protoplasts isolated from the salt-stressed leaves had a much lower level of intactness. To try to determine at what stage in the isolation procedure the

T A B L E XXVI

Percentage Intactness of Barley Protoplasts Isolated From Control
Leaves and From Leaves Stressed by 300mM Mannitol and 400mM NaCl

~ 200 protoplasts were tested in each case.

Protoplast Sample	Intactness Before Centrifugation	Intactness After First Centrifugation	Intactness After Third Centrifugation (At the end of the isolation)
Control	-	-	92%
300mM Mannitol	-	-	90%
400mM NaCl	94%	81%	63%

protoplasts from salt-stressed leaves were becoming damaged, their percentage intactness was tested immediately after the incubation period (before any centrifugation), and after the first centrifugation. These results (Table XXVI) were then compared to the percentage intactness of the protoplasts at the end of the isolation procedure (after the third centrifugation).

The results suggest that, unlike protoplasts from the control and mannitol-stressed leaves, protoplasts from the salt-stressed leaves had been damaged severely by centrifugation during the isolation procedure.

5.1.2 Oxygen Evolution

The steady-state oxygen evolution rates of barley protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol are shown in Table XXVII. This table also compares the oxygen evolution rates of these protoplasts with the rates obtained previously for the corresponding control and mannitol-stressed leaves (Section 4.4.4).

Although both sets of protoplasts showed lower rates of oxygen evolution than the corresponding leaves, mannitol decreased the rates of oxygen evolution by approximately the same amount in both experimental systems (19% decrease with protoplasts; 24% decrease with leaves).

It proved impossible to calculate an accurate rate of oxygen evolution for protoplasts isolated from 400mM NaCl-stressed leaves because of the unstable response of the oxygen electrode when measurement was attempted. Presumably, as suggested by the results of the intactness tests (Section 5.1.1), these protoplasts were damaged even more severely by the stirrer of

T A B L E XXVII

Steady-State Oxygen Evolution Rates of Barley Protoplasts Isolated From Control Leaves and From Leaves Stressed by 300mM Mannitol

Protoplast assays were carried out at 25°C and at a final chlorophyll concentration of $10\mu\text{g}.\text{ml}^{-1}$ in a medium containing 500mM mannitol, 1mM CaCl_2 , 5mM Tricine (pH 7.6) and 5mM KHCO_3 . The protoplasts were illuminated by broad-band blue light (Corning 4.76, Corning 4.96 and Calflex C filters) of intensity 158Wm^{-2} . The table also shows the oxygen evolution rates previously obtained (Section 4.4.4) for the corresponding control and stressed leaves. Results indicate the mean \pm S.E.M. for the number of determinations shown in parentheses.

Sample	Oxygen Evolution Rate ($\mu\text{moles O}_2.(\text{mg chl})^{-1}.\text{h}^{-1}$)	
	Protoplast System	Leaf System
Control	106 \pm 6.4 (3)	225 \pm 23.1 (7)
300mM Mannitol	86 \pm 7.7 (3)	170 \pm 16.0 (7)

the oxygen electrode, resulting in very low rates of oxygen evolution.

5.1.3 Chlorophyll Fluorescence

Figure 47 shows the typical chlorophyll fluorescence curves which occur during the induction period of barley protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol and 400mM NaCl. Table XXVIII shows the height of the S-M transient for each set of protoplasts, and compares these heights with those obtained previously for the corresponding control and stressed leaves (Section 4.4.5).

Protoplasts isolated from the control and mannitol-stressed leaves showed similar induction curves to those of the corresponding leaves : both sets of protoplasts had distinct P, S, M and T phases. These phases were still distinct more than five hours after the protoplasts had been isolated. Although the S-M transients of both sets of protoplasts were much lower than those of the corresponding leaves, mannitol decreased the height of the S-M transient in both experimental systems.

In contrast, protoplasts isolated from the salt-stressed leaves showed induction curves which were unlike those of the corresponding leaves. After the P peak, there was very little quenching of chlorophyll fluorescence, and there was no distinct S or M phase.

5.1.4 DCMU-Induced Relaxation of Chlorophyll Fluorescence Quenching

Sections 5.1.1 to 5.1.3 have shown that barley protoplasts isolated from control leaves and from leaves stressed by 300mM

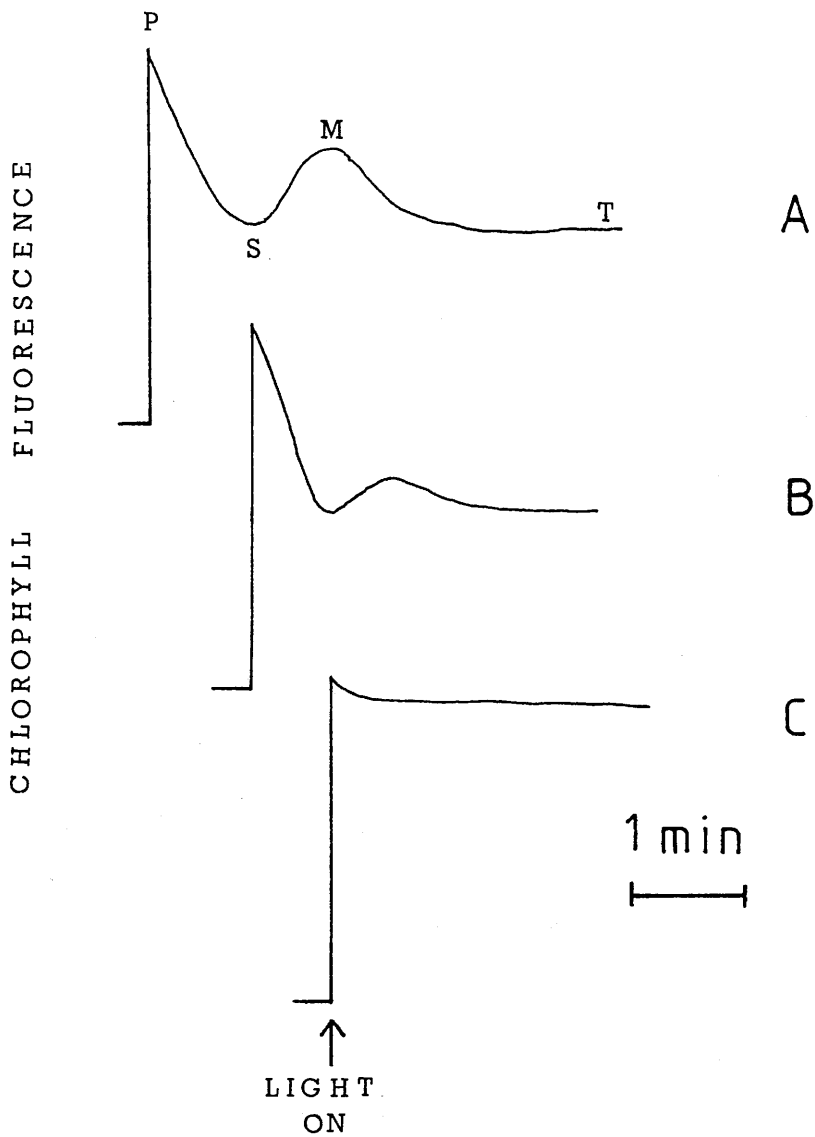


Figure 47. Typical chlorophyll fluorescence curves during the induction period of barley protoplasts isolated from A) control leaves; B) 300mM mannitol-stressed leaves and C) 400mM NaCl-stressed leaves. The protoplasts were dark-adapted for 10 minutes prior to illumination with broad-band blue light (Corning 4.76, Corning 4.96 and Calflex C filters) of intensity 158Wm^{-2} . Assays were carried out at 25°C and at a final chlorophyll concentration of $10\mu\text{g}\cdot\text{ml}^{-1}$ in a medium containing 500mM mannitol, 1mM CaCl_2 , 5mM Tricine (pH 7.6) and 5mM KHCO_3 . Each curve is representative of an experiment which was replicated at least five times.

T A B L E XXVIII

Height of the S-M Chlorophyll Fluorescence Transient, Relative to the Height of the P Peak, in Barley Protoplasts Isolated from Control Leaves and From Leaves Stressed by 300mM Mannitol and 400mM NaCl

Protoplast assays were carried out at 25°C and at a final chlorophyll concentration of 10 μ g.ml⁻¹ in a medium containing 500mM mannitol, 1mM CaCl₂, 5mM Tricine (pH 7.6) and 5mM KHCO₃. The protoplasts were illuminated by broad-band blue light (Corning 4.76, Corning 4.96 and Calflex C filters) of intensity 158Wm⁻². The table also shows the heights of the S-M transients previously obtained (Section 4.4.5) for the corresponding control and stressed leaves. Results indicate the mean \pm S.E.M. for the number of determinations shown in parentheses.

Sample	Height of the S-M Transient, Relative to the Height of the P Peak (Relative Units)	
	Protoplast System	Leaf System
Control	3.1 \pm 0.56 (8)	13.5 \pm 1.06 (16)
300mM Mannitol	2.3 \pm 0.33 (8)	5.7 \pm 1.20 (16)
400mM NaCl	No distinct S or M transient	2.2 \pm 0.76 (16)

mannitol had a high level of intactness at the end of the isolation procedure, reasonable rates of oxygen evolution and chlorophyll fluorescence transients similar to those of the corresponding control and mannitol-stressed leaves. This suggests that the metabolism of these protoplasts is related to that of the leaves. Consequently, these protoplasts should be useful experimental systems in which to estimate q_e by DCMU-induced relaxation of chlorophyll fluorescence quenching.

However, protoplasts isolated from leaves stressed by 400mM NaCl were much less intact, had low rates of oxygen evolution and had chlorophyll fluorescence transients unlike those of the salt-stressed leaves. These protoplasts would obviously not be of use in estimating q_e by DCMU addition. Therefore, DCMU was added only to protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol. The DCMU (10 μ M) was added at the points S, M and T on the induction curve (using separate protoplast samples in each case) to determine the way in which q_e changed during the induction period. Values for q_Q were also estimated to determine how they compared to the values of q_Q previously obtained for leaves using light doubling (Section 4.4.6).

Figure 48 shows the biphasic increase in chlorophyll fluorescence which occurred when DCMU was added at T to protoplasts from control leaves. The extent of the fast phase (R_f) was expressed as a percentage of the chlorophyll fluorescence before the DCMU addition. The extent of the slow phase (R_s) was expressed as a percentage of the chlorophyll fluorescence level before the DCMU addition plus the amount of chlorophyll fluorescence attributed to the fast phase. R_f and R_s were expressed in the same way for protoplasts from the

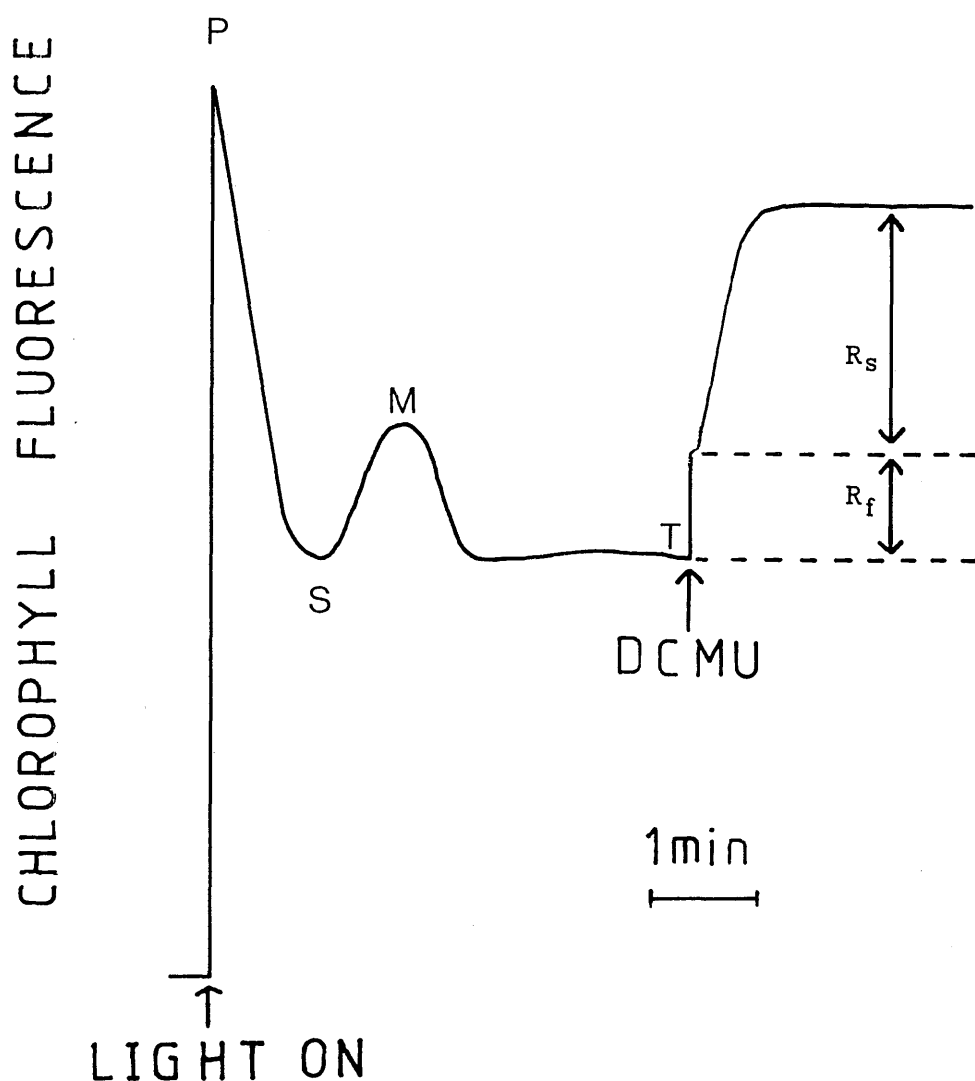


Figure 48. The effect of addition of $10\mu\text{M}$ DCMU at T on quenching in barley protoplasts isolated from control leaves. R_f and R_s represent the fast and slow phases of the increase in chlorophyll fluorescence, respectively, on addition of DCMU. The protoplasts were dark-adapted for 10 minutes prior to illumination with broad-band blue light (Corning 4.76, Corning 4.96 and Calflex C filters) of intensity 158Wm^{-2} . Assays were carried out at 25°C and at a final chlorophyll concentration of $10\mu\text{g.ml}^{-1}$ in a medium containing 500mM mannitol, 1mM CaCl_2 , 5mM Tricine (pH 7.6) and 5mM KHCO_3 .

mannitol-stressed leaves. The values of R_f and R_s were taken to represent the values for q_Q and q_e respectively (Table XXIX).

The values for q_Q obtained by DCMU addition to both sets of protoplasts were lower than those obtained by light doubling in leaves (Section 4.4.6). However, light doubling had shown a slight tendency for Q to be more reduced in the mannitol-stressed leaves than in the control leaves during P to S quenching. Similarly, Q was slightly more reduced at S in protoplasts from the mannitol-stressed leaves than in protoplasts from control leaves.

The value of q_e in protoplasts from both control and mannitol-stressed leaves was highest at the S level; it then decreased at M and continued to decrease to T. There was no obvious difference in the value of q_e between the two sets of protoplasts.

5.1.5 Discussion

Centrifugation during the isolation procedure appeared to damage barley protoplasts from leaves stressed by 400mM NaCl. Perhaps the presence of a high concentration of Na^+ or Cl^- ions in these protoplasts had altered their buoyancy so that they were subjected to shear forces as they moved through the sucrose-mannitol gradient.

There was no difference in the values for q_e , nor in the way q_e changed during the induction period, between barley protoplasts isolated from control leaves and from leaves stressed by 300mM mannitol. The value of q_e was highest at S, lower at M and lower still at T. This was expected from the theory of

T A B L E XXIX

Measurement of % q_Q and % q_e at S, M and T on the Chlorophyll
Fluorescence Induction Curves of Protoplasts Isolated from Control
Leaves and from Leaves Stressed by 300mM Mannitol

Results indicate the mean \pm S.E.M. for 8 determinations.

Stressing Solution	% q_Q			% q_e		
	S	M	T	S	M	T
Control	20 \pm 2.1	17 \pm 1.8	43 \pm 3.3	32 \pm 2.5	24 \pm 1.9	19 \pm 2.7
300mM Mannitol	14 \pm 2.4	15 \pm 1.8	43 \pm 6.2	29 \pm 3.8	19 \pm 3.0	17 \pm 4.1

quenching during the induction period (Section 1.8.1). Fast electron transport from P to S leads to the build-up of a large transmembrane proton concentration gradient and, consequently, to a high value for q_e at S. After S, carbon dioxide fixation is thought to dissipate the transmembrane proton concentration gradient by consumption of ATP, resulting in a decrease in q_e at M. The value of q_e continues to decrease to T as the rate of carbon dioxide fixation accelerates to its maximum rate.

C H A P T E R 6

GENERAL DISCUSSION

The results obtained in Chapters 3 to 5 will be discussed in this chapter in an attempt to answer the three questions posed at the beginning of this thesis : (i) to what extent does NaCl stress affect photosynthesis; (ii) which of the underlying mechanisms in the photosynthetic process are affected by NaCl and (iii) is the effect of NaCl toxic, osmotic or both?

6.1 The Effect of NaCl on Photosynthesis

The effect of NaCl on photosynthesis in barley was studied by examining the oxygen evolution rates and chlorophyll fluorescence induction kinetics of control and salt-stressed leaf pieces.

NaCl was found to inhibit severely the oxygen evolution rates of barley leaf pieces (Section 3.4.3). Some previous studies have concluded that the decline of photosynthesis in response to increased salinity is to some extent the result of decreased stomatal conductance (Longstreth and Nobel, 1979; Robinson, Downton and Millhouse, 1983). The results obtained in Section 3.4.1 showed that, under atmospheric concentrations of carbon dioxide, there was a salt-induced decrease in the stomatal conductance of carbon dioxide which, under these conditions, would be expected to decrease the oxygen evolution rates of the salt-stressed leaf pieces compared with those of the control leaf pieces. However, a salt-induced decrease in oxygen evolution was still observed when both control and salt-stressed

leaf pieces had the same, fast rates of carbon dioxide intake (in the chamber of the Leaf Disc Electrode). This suggests that NaCl must also inhibit oxygen evolution by some method which does not involve stomata. It seems likely that in the field, NaCl inhibits oxygen evolution in barley at the biochemical level as well as at the level of the stomata.

The major effect of NaCl on the chlorophyll fluorescence induction kinetics of barley leaf pieces was to decrease the height (increase the quenching) of the S-M transient (Section 3.5). There was no consistent difference in the rate of chlorophyll fluorescence quenching (slope of decline) from P to S between control and salt-stressed leaf pieces. In contrast, Downton and Millhouse (1985) have observed an increase in the rate of chlorophyll fluorescence quenching from P to S as a result of salt stress in barley, but their study did not examine quenching during the S-M-T phase of chlorophyll fluorescence.

6.2 The Effect of NaCl on Underlying Photosynthetic Processes

The underlying photosynthetic processes which NaCl affects to bring about the changes in oxygen evolution and chlorophyll fluorescence described above were investigated using modulated chlorophyll fluorimetry; this technique enabled a study to be made of NaCl effects on the redox state of Q during induction (Section 3.6), and on changes in light energy distribution between the two photosystems (Section 3.7). The results suggested that NaCl inhibited the transition from State 1 to State 2 under conditions of over-excitation of PSII. This caused an increase in the activity of PSII at the expense of PSI and led to a more reduced state of Q. The imbalance in excitation energy

entering the two photosystems may explain the decrease in oxygen evolution with increasing [NaCl]; maximum rates of electron transport require the simultaneous and equal operation of the two photosystems.

Similar changes in the distribution of excitation energy between the two photosystems as a result of salt stress have been observed in isolated chloroplasts (Barber, 1976) and thylakoids (Dominy and Baker, 1980) and are thought to be the result of conformational changes in the thylakoid membrane. In isolated thylakoids, cations are thought to induce changes in the electrical double layer at the interface of the outer surface of the thylakoid and the solution, which result in changes in both the membrane surface potential and the electrical potential difference across the membrane (Mills and Barber, 1978). Cations may also produce increased hydrophobic interactions within the thylakoid membrane, which result in changes in water-lipid interfaces (Papageorgiou, 1975). Such conformational changes would be expected to interfere with the arrangement of the components of the photochemical apparatus and induce changes in photochemistry and light energy distribution.

While conformational changes in the thylakoid membrane may explain the salt-induced inhibition in the transition from State 1 to State 2 which was observed here for barley, this study has not determined whether the Na^+ ions which are taken into barley leaves actually enter the chloroplasts and exert a direct ionic effect on the thylakoid membrane. Ion concentrations in chloroplasts are very difficult to measure accurately because redistribution of ions may occur during the isolation procedure; estimates of chloroplast cation concentrations have ranged from 3 to 550mM for Na^+ , 80 - 400mM for K^+ and 30 - 110mM for Mg^{2+}

(Harvey and Brown, 1969; Nobel, 1969; Murakami, Torres-Pereira and Packer, 1975). However, even if Na^+ ions do not enter barley chloroplasts, the accumulation of Na^+ in barley leaves may still affect thylakoid function by inducing changes in chloroplast water relations.

Measurement of q_Q during the chlorophyll fluorescence induction curves of control and salt-stressed barley (Section 3.6) suggested that the increased quenching of the S-M transient in salt-stressed barley was the result of an increase in one of the quenching mechanisms other than q_Q . To examine the possibility that this other quenching mechanism might be q_e , an attempt was made to study the effect of NaCl on q_e by adding DCMU to protoplasts which had been isolated from control and salt-stressed leaves (Section 5.1). However, this proved unsuccessful because the protoplasts isolated from the salt-stressed leaves were damaged by centrifugation during the isolation procedure. Perhaps a better method would be to add DCMU to individual cells isolated from salt-stressed leaves. These cells could be isolated by the same method as was used for protoplasts except that barley leaf segments would be incubated with pectinase only, rather than cellulase and pectinase. The presence of cell walls would make these cells much less liable to damage during isolation but should still allow the rapid penetration of DCMU. Individual cells have been used successfully in herbicide research to study the effect of herbicides such as DCMU on whole cell metabolism (Pallett et al, 1986).

6.3 The Use of Mannitol to Distinguish Between Toxic and Osmotic Effects of NaCl

Mannitol was chosen as the substance with which to stress barley osmotically. However, mannitol was not excluded completely from barley leaves (Section 4.3.2) so that the possibility of it exerting physiological effects cannot be discounted. Nevertheless, comparison of the effects of NaCl - and mannitol - stressing solutions which induced the same leaf water potential showed that toxic effects of NaCl were greater than osmotic effects of NaCl in all of the parameters studied. Another piece of evidence for this greater toxic effect of NaCl is that although the leaf water potential recovered slightly at the highest external NaCl concentrations, all of the photosynthetic parameters were affected most severely by the highest external NaCl concentrations.

6.4 Future Implications of Salt Stress for Agriculture

The threat posed by the progressive build-up of salt has traditionally led to the creation of large schemes of reclamation and drainage. In addition, irrigation schemes have been set up; these use high-quality water which often has to be transported long distances. Such schemes minimise the problem of salinity but cannot eliminate it, and the need nowadays is to supplement these schemes with the genetic development of salt-tolerant crops (Epstein et al., 1980). The selection and breeding of salt-tolerant strains could improve the use of saline soils and saline water. For instance, a saline soil might not support the growth of certain species but it might allow the normal growth of

selected salt-tolerant strains of those same species. In addition, the use of saline water for irrigating salt-tolerant strains would make high-quality water available for the irrigation of salt-sensitive species and for domestic use.

Plants can be screened for salt-tolerance by noting their lack of visible salt-induced symptoms such as leaf burn. However, such symptoms are rather late signs of severe salt stress and are thus of limited use in screening for salt tolerance. Recently, Smillie and Nott (1982) have discussed the advantages of using chlorophyll fluorescence as a screening technique. As well as being rapid and non-destructive, measurements of chlorophyll fluorescence can detect adverse effects of salt before these effects cause visible symptoms and reduce the yield of field-grown crops. In barley, the S-M chlorophyll fluorescence transient may be an early indicator of stress since it was much more affected by osmotic stress alone than were any of the other parameters (Section 4.4.8). A decrease in the height of S-M may be the first warning sign that barley is experiencing salt stress. Such changes in the chlorophyll fluorescence transients can now be detected in the field using a recently-developed, portable instrument (Ögren and Baker, 1985). This instrument can also be used in the field to monitor the State 1 - State 2 transitions which are assumed to occur naturally when the spectral composition changes in the morning and evening. In the future, the effects of NaCl on these natural State 1 - State 2 transitions in barley could be compared with the effects of NaCl on the State 1 - State 2 transitions observed in this study (Section 3.7).

6.5 Conclusions

This study has shown that photosynthesis in barley is affected severely by high external NaCl concentrations. One of the most important effects of NaCl is to inhibit the transition from State 1 to State 2 under conditions of over-excitation of PSII. Although the effect of NaCl was mostly toxic, osmotic effects were also involved to some extent.

A P P E N D I X

Knopp's nutrient solution was made up in distilled water as below :

KCl	4 mM
MgSO ₄ ·7H ₂ O	2 mM
Ca(NO ₃) ₂ ·4H ₂ O	2 mM
KH ₂ PO ₄	2 mM
FeC ₆ H ₅ O ₇ ·5H ₂ O	90 μM
H ₃ BO ₃	10 μM
KMnO ₄	3 μM
MnCl ₂ ·4H ₂ O	2 μM
CoCl ₂	0.4 μM
ZnSO ₄ ·7H ₂ O	0.3 μM
CuSO ₄ ·5H ₂ O	0.3 μM
KBr	0.3 μM
KI	0.2 μM
SnCl ₂	0.2 μM
Li ₂ SO ₄ ·H ₂ O	0.3 μM
Al ₂ (SO ₄) ₃	0.2 μM
NiSO ₄ ·6H ₂ O	0.2 μM
Na ₂ MoO ₄ ·2H ₂ O	0.1 μM

R E F E R E N C E S

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